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**Interplay Between Endocannabinoid System and Neurodegeneration: Focus on Polypharmacology**

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**ABSTRACT**

Pharmacological treatment of complex pathologies such as neurodegenerative diseases still represents a major challenge, due to the networked pathways involved in their onset and progression that may require equally complex therapeutic approaches. Polypharmacology, based on the simultaneous modulation of multiple targets involved in the disease, may offer the potential to increase effectiveness and reduce the drawbacks related to the use of drug combinations. Clearly, this approach requires both the knowledge of the systems responsible for disease development and the discovery of new attractive targets to be exploited to design a multitarget drug. Over the last years, an ever increasing interest has focused on the endocannabinoid system, implicated in the modulation of several physiological functions, among which neuroinflammation, a crucial process for most neurodegenerative diseases. In this respect, the cannabinoid receptor subtype 2 represents a promising therapeutic target, being overexpressed in microglia cells and thus involved in neuroinflammation. The indirect modulation of this system through the inhibition of the main enzymes responsible for endocannabinoids metabolism, namely fatty acid amide hydrolase and monoacylglycerol lipase, may also significantly affect neurodegenerative processes. The aim of this review is to give an overview of the opportunities posed by the endocannabinoid system for neurodegenerative diseases management, mainly focusing on the potential for a multitarget strategy.

**KEYWORDS:** Endocannabinoid System, Neurodegenerative diseases, multitarget drug, Alzheimer's Disease, Parkinson's Disease, cannabinoid receptors, FAAH, MAGL.

## 1. INTRODUCTION

The endocannabinoid system (ECS) is involved in the modulation of numerous physiological functions, involving, among others, pain, motor function, immune system, and cognition, and is acquiring an

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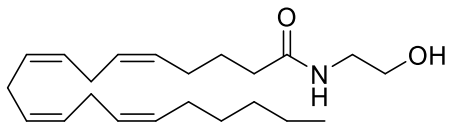
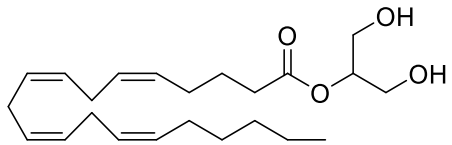
increasingly prominent role as a central nervous system (CNS) regulator of both synaptic transmission and plasticity. Moreover, it may also be responsible for exerting neuroprotective effects following neuronal injury, while, on the other hand, its dysregulation could trigger pathological conditions, including neurological diseases [1, 2].

Endogenous cannabinoids (eCBs), also referred as endocannabinoids, represent the endogenous neurotransmitters of the ECS and are synthesised on demand from lipid membrane constituents. Anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) (Figure 1) are the most studied and the best characterized eCBs and exert their biological activities by interacting with two primary G protein-coupled receptors (GPCRs): cannabinoid 1 receptor (CB1R) and cannabinoid 2 receptor (CB2R). CB1R is widely expressed in the CNS, both in neurons and glial cells, where it modulates excitatory and inhibitory neurotransmission and regulates neurotransmitters release [3]. It is mainly found in the prefrontal cortex, hippocampus, caudate, putamen, nucleus accumbens, and striatum [4], while CB2R is predominantly located in peripheral cells and tissues derived from the immune system, such as macrophages, B-cells and natural killer cells. CB2R has been also found in activated microglia [5], the resident CNS phagocytes of the immune system, responsible for the immune response to CNS damage by releasing proinflammatory cytokines. The involvement of CB2R in microglia-derived neuroinflammation has been clearly recognized and the role of this receptor in modulating distinct factors of CNS inflammatory response, comprising microglial cell proliferation and migration, makes it a promising pharmacological target for neuroprotection [6]. Activated microglia have been grouped in at least two states endowed with contrasting roles, namely M1 (pro-inflammatory) and M2 (anti-inflammatory), even if recent studies lean toward the simultaneous existence of multiple microglial phenotypes involved in neuroinflammation. When microglia become activated into a protective phenotype, the production of 2-AG and AEA increases significantly, leading to the activation of CB1 and CB2 receptors and their signalling cascades, further intensifying the anti-inflammatory microglial phenotype [7].




**Anandamide (AEA)**




**2-arachidonoyl glycerol (2-AG)**

**Figure 1.** Most studied endocannabinoids

ECS also comprises other 7-transmembrane receptors, among which GPR55 (“supposed CB3”), nuclear receptors, namely peroxisome proliferator-activated receptors (PPARα, PPARβ/δ and PPARγ), transient receptor potential cation channel subfamily V member 1 (TRPV1) [8, 9] and the G-protein-coupled receptor GPR18, belonging to the orphan class A family, for which a relationship with the endocannabinoid system has been recently proved [10].

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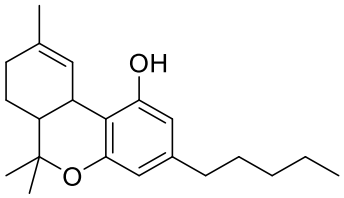
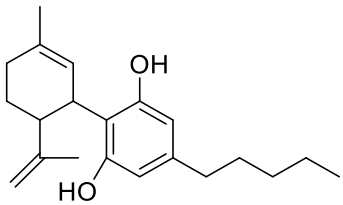
A pivotal role in regulating eCBs activity is also to be attributed both to the synthesizing enzymes, N-acyl-phosphatidylethanolamine-phospholipase D (NAPE-PLD) and diacylglycerol lipase (DAGL), and to the main degradative enzymes, namely fatty acid amide hydrolase (FAAH), responsible for AEA inactivation, and monoacylglycerol lipase (MAGL), involved in 2-AG degradation. [4]. The latter metabolizing enzymes belong to the family of serine hydrolases, are highly expressed in both the CNS and in peripheral tissues, and show broad substrate selectivity.

Recently, the interconnection between ECS and other neurotransmission systems, including the glutamate N-methyl-D-aspartate receptor (NMDAR), a key molecular tool for controlling synaptic plasticity and memory, and the dopaminergic mesolimbic system, has been widely evaluated [11, 12]. In particular, as for others GPCRs, the ability of cannabinoid receptors (CBRs) to form physiologically interacting homodimers of the same receptor subtype or heterodimers of two different receptors has been observed, witnessing the crosstalk between apparently unrelated systems. For CB1R, interactions with CB2R, opioid receptors, dopamine D2 receptor, β2 adrenergic receptor, adenosine A2A receptor, and angiotensin II AT1 receptor were reported. Both CBRs can also form heterodimers with the orphan GPR55 receptor [13].

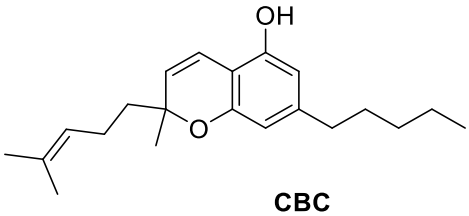
The term phytocannabinoid describes natural-derived polyphenolic compounds decorated with an alkyl, aralkyl, or isoprenyl side chain [14], whose activities overlap with eCBs, due to their binding to the same receptors. *Cannabis sativa* represents the most studied source of phytocannabinoids and contains more than a hundred different cannabinoids, classified based on their peculiar structure. The main constituents are trans-Δ9-tetrahydrocannabinol (THC), cannabidiol (CBD), cannabichromene (CBC) and cannabigerol (CBG) (Figure 2). While THC is the well-known psychoactive component found in marijuana and a nonselective CBRs agonist, CBD has recently attracted great interest, being devoid of euphorigenic or psychedelic properties and showing the ability to regulate neurotransmitters release by acting on 5-hydroxytryptamine receptors, TRP-like channels and PPARγ receptors [1, 14]. Recently, the modulation of CBRs by binding to an allosteric site has been reported for CBD and all these effects may be involved in its recognized neuroprotective activity. A review reporting the therapeutic potential of CBD and its analogues in epilepsy and Alzheimer's disease (AD) has been recently published [15].



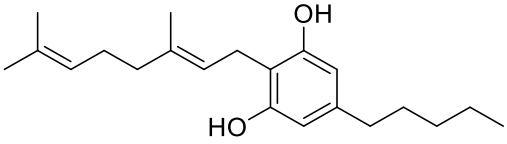

**Δ-9-THC**




**CBD**




**CBC**




**CBG**

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**Figure 2.** Representative phytocannabinoids.

This broad-spectrum profile, involving multiple molecular sites of action, within and outside the ECS, can be considered the main advantage of eCBs in neuroprotection, pinpointing the potential of ECS-targeted drugs to act as disease-modifying therapy for facing still untreatable diseases.

Neurodegenerative diseases (NDs) are a heterogeneous group of disorders characterized by the progressive relapse of neuronal structure and function in distinct brain regions, accounting for diverse clinical manifestations. However, common features and mechanisms can be recognized in different NDs, in particular the formation of cytosolic or nuclear proteins aggregates spreading from one region to another during disease progression [16]. All NDs recognize aging as the main risk factor, and an increase in the prevalence of these devastating diseases appears unavoidable, due to the rising of life expectancy worldwide. The most common NDs are AD and Parkinson's disease (PD), but Huntington's disease (HD), multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS) are also widely studied and monitored. The aetiology of these diseases is far from being completely elucidated, and a number of different pathways and factors seem to be networked in their onset and progression, among which neuroinflammation appears as a common feature embroiled in the pathogenesis of functional and mental impairments [17]. In such a complex framework, modern drug discovery has developed a polypharmacology approach, based on the design of a single molecule able to engage different targets/pathways involved in the disorder [18], and ECS may represent a suitable candidate in a multitarget perspective. Indeed, a modulation of ECS can be attained by designing specific CBRs ligands or acting by inhibiting FAAH and MAGL, the enzymes responsible for the deactivation of the eCBs AEA and 2-AG, unveiling various potential targets to be exploited.

This review aims at illustrating the diverse opportunities offered by the ECS for treating NDs, mainly focusing on the pleiotropic effects of this system and the resulting potential for a multitarget approach.

## 2. ECS AND NEURODEGENERATIVE DISEASES

As abovementioned, AD and PD are the prevailing NDs and are worldwide recognized as the leading cause of dementia. AD is a progressive ND, exponentially related to aging and accounting for more than 50% of all cases of dementia. [19]. The main pathognomonic hallmarks of the disease are a progressive loss of forebrain cholinergic neurons [20], the formation of neuritic plaques due to amyloid-beta (Aβ) aggregation and deposition [21], the hyperphosphorylation of tau protein associated with neurofibrillary tangles formation [22], together with neuroinflammation sustained by activated microglia [7]. In this complex framework, the role of ECS is acquiring ever increasing interest. To date, the involvement of CB1R in this disease remains somewhat unclear. The expression of this receptor appears time-dependent and associated with the progressing stages of the neurodegenerative process [23, 9]. Various studies described an increased CB1R expression in the earliest stages of the disease, mainly in limited hippocampal areas and in the frontal cortex, while recent experiments in transgenic mouse models reported a decreased hippocampal expression of this receptor associated with neuronal loss, leading to speculating an involvement of CB1R during disease progression [24, 25]. This receptor has been implicated in protection against cell death induced by excitotoxicity, and its increased activity during the initial stages of AD may represent a defending strategy of ECS in response to the primary neuronal insult [26]. However, the persistent loss of neurons expressing CB1Rs during neurodegenerative processes and the concomitant psychotropic effects associated with the administration of CB1R agonists make this receptor an intricate

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target to be exploited for neuroprotection [27]. Conversely, the involvement of CB2R in AD pathology is supported by a relevant number of studies. Indeed, in postmortem AD brains, a correlation between CB2R upregulation and Aβ-enriched plaques has been noticed, and CB2R expression considerably increases in reactive microglia and astrocytes during neuroinflammation, probably in an attempt to mitigate the inflammatory state. This peculiar location enables compounds targeting CB2R to keep a selective control on the specific functions monitored by these cells in degeneration processes [28] without associated psychotropic effects. In transgenic mouse models of AD, CB2 agonist administration appears to improve cognitive performance, mainly tempering cytokine release and promoting amyloid clearance [29, 30].

Promising therapeutic advantages by exploiting ECS and CBRs-interacting compounds were also reported to treat PD symptoms, such as tremors and dyskinesia [31, 32]. PD is a progressive ND, sharing common features with AD and other neurodegenerative conditions. Similar to AD, it is characterised by the progressive loss of neurons and the formation of aggregates of misfolded proteins, in this case affecting the dopaminergic system in the basal ganglia and involving the formation of Lewy bodies within the cell, generated by aberrant α-synuclein aggregates. Therefore, the typical symptoms of PD pertain to motor functions and include dyskinesia, muscle rigidity, postural instability, and tremors, associated with nonmotor dysfunctions, among which cognitive impairment emerges as the most disabling [33]. As in AD, neuroinflammation plays a pivotal role in PD pathogenesis, and concurrent microglia activation significantly correlates with upregulation of CB2Rs [34]. In this respect, overactivity of ECS has been observed and widely studied in PD patients and in animal models of the disease, being also CB1R abundantly expressed in basal ganglia, indicating a possible involvement in motor function regulation [6, 35].

Besides AD and PD, other NDs typified by neuroinflammatory conditions, such as HD, ALS, and MS, involve the ECS and could benefit from its appropriate modulation [36].

*2.1 CB2 receptor modulation: different operating modes*

With respect to CB1R, brain CB2R exhibits some peculiar features that make it a preferable target for neuroprotection, among which its inability to mediate the psychotropic effects of *cannabis*, its peculiar distribution and enhanced expression under some pathological conditions (i.e. inflammatory state), suggesting a close connection between this receptor and neurological diseases [6]. In recent years, several studies have been devoted to the identification of novel CB2 agonists devoid of CB1 central effects to discover high-affinity and selective compounds aimed at facing different pathologies involving a neuroinflammatory component, such as NDs, but also including pain perception, ischemic stroke, autoimmune diseases, osteoporosis, and various cancer types [37]. The development of drugs acting on CB2R appears very challenging, and only a small number of synthetic CB2 agonists have reached clinical trials, mainly for treating pain conditions [38].

Notably, CB2 agonists proved to differently interact with the receptor site, suggesting diverse functional properties that allow them to be classified as full, partial, and inverse agonists [37]. Interestingly, an antagonist/inverse agonist may also possess anti-inflammatory activity [39], paving the way for an additional option to deal with this receptor. In the last decade, the search for CB2R ligands has received great interest both by industry and academia, leading to the identification of diverse compounds and structural scaffolds endowed with CB2R interacting properties, commonly lipophilic molecules embodying aromatic heterocycles linked to bulky aryl or alkyl fragments. The structure-activity relationship (SAR) and structure-affinity relationship (SAfiR) have been recently reviewed, mainly devoted to the discovery of new pain therapies [40, 41]. Clearly, the definition of the effective functional operating mode following receptor

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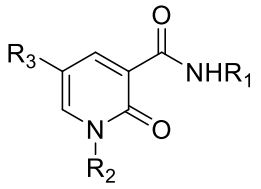
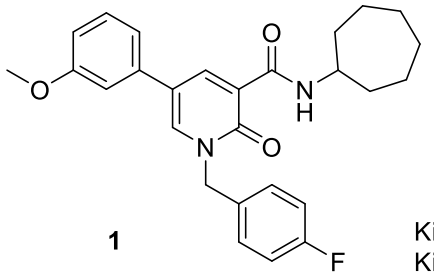
binding acquires a pivotal role to better define the role of ECS in pathological conditions and to identify the therapeutic suitability of a CBR ligand.

In the context of a research program involving the development of CB2R selective ligands, the group of C. Manera reported a study devoted at improving the ability of their previously discovered 1,2-dihydro-2-oxopyridine-3-carboxamide core to modulate CB2R [42]. In particular, compound **1** (Figure 3) showed a very high affinity for CB2R and a significant selectivity towards CB1R. Interestingly, this compound also proved to act as a potent CB2R neutral antagonist/weak partial inverse agonist. Moreover, these researchers also found that the functional activity of these compounds was depending on the nature of the 5-substituent of the 1,2-dihydro-2-oxopyridine nucleus: replacing the hydrogen with a phenyl in this position led to a shift of CB2R activity from agonism to inverse agonism, while introducing a *para*-methoxyphenyl group in this position gave rise to a CB2R neutral antagonist/weak partial inverse agonist. The CB2R neutral antagonist behaviour of these compounds may be useful for a better understanding of the role of this receptor, and a further study was then pursued by the same group [43], leading to the identification of a biphenyl carboxamide analogue as a potent and selective CB2R ligand (**2**, Figure 3) showing a pharmacological profile attributable to CB2 receptor selective neutral antagonism.

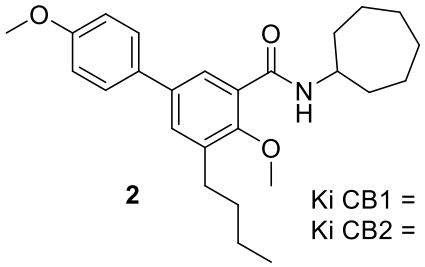



1,2-dihydro-2-oxopyridine-3-carboxamide derivatives: general formula



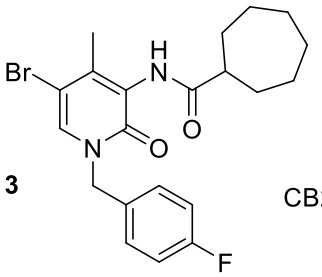

**1**

Ki CB1 = 63 nM
Ki CB2 = 1.0 nM




**2**

Ki CB1 = 1494 nM
Ki CB2 = 11.48 nM




**3**

CB2R Positive allosteric modulator

**Figure 3**. 2-oxopyridine-3-carboxamides and related compounds developed by the Manera group.

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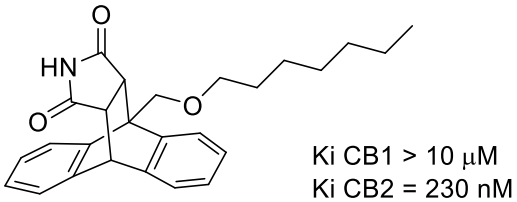
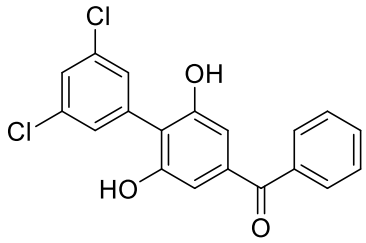
Finally, by modifying the amide function of their orthosteric CB2R ligands, in 2019, compound **3** (Figure 3) was discovered as the first positive allosteric modulator targeting CB2R, that could represent a valuable therapeutic option to treat neuropathic pain, avoiding potential side effects related to orthosteric binding [44]. Allosteric modulators, interacting with sites different from the orthosteric ligand-binding domain, induce a conformational modification of the receptor, modulating the activity of orthosteric ligands without receptor activation. In a following paper, the same group also demonstrated that a combination of **3** with a dual orthosteric agonist of CBRs might synergistically act towards neuroinflammation in NDs [45]. Taken together, all these data pinpoint the 2-oxo-pyridine-3-carboxamide scaffold as a leading core structure for ECS modulation.

On the other hand, in the same years, our research group reported a series of CBRs ligands bearing a previously unexplored polycyclic scaffold (**4**, Figure 4) showing appreciable affinity and significant selectivity for CB2R, endowed with a noncompetitive antagonist activity. Due to the easily affordable and modifiable chemical structure, these compounds could represent useful chemical tools for a better understanding of the ECS impact on several diseases. [46]



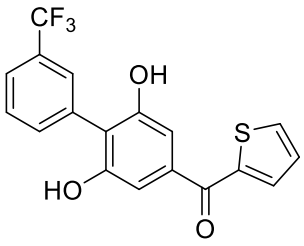

**4**

Ki CB1 > 10 μM
Ki CB2 = 230 nM




**SMM-189**

Ki CB1 = 4778 nM
Ki CB2 = 121 nM




**5**

Ki CB1 > 10000 nM
Ki CB2 = 154 nM

**Figure 4.** Recently reported modulators of CB2R with innovative scaffolds.

Very recently, Alghamdi *et al*. [47] reported a comprehensive SAR study on the selective CB2 inverse agonist SMM-189 (Figure 4), a 2,6-dihydroxy-biphenyl-aryl-methanone derivative developed in their laboratories able to promote *in vitro* switching from the microglia M1 proinflammatory phenotype to the M2 pro-healing phenotype and showing a promising *in vivo* activity as neuroprotective and anti-inflammatory agent. From this study, compound **5** (Figure 4) emerged as a potent and effective CB2 inverse

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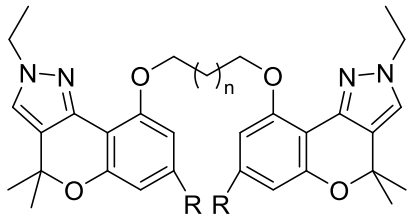
agonist, providing further evidence for validating the 2,6-dihydroxy-biphenyl-aryl-methanone scaffold as a modulator of microglia activation.

A convenient medicinal chemistry strategy to increase selectivity versus CB1R is the design of bitopic ligands, single molecular entities able to interact with both the orthosteric site and a less conserved site within the same receptor, called exosite. It is usually located at the entrance of the orthosteric site, and the simultaneous engagement of both sites may lead to an improved selectivity and bioavailability [48]. However, the design of bitopic ligands for lipid CBRs appears very challenging [49], mainly due to the peculiar disposition of the binding sites, embedded in the lipid bilayer membrane. In 2020, the group of Nadine Jagerovic reported the first bitopic ligands for CB2R, rationally designed on the basis of the recently released structure of CB2R in its active Gi-bound conformation [50], in order to properly define the binding mode of the ligands [51]. The chromeno-pyrazole core, previously identified by the group as CB2R orthosteric agonists, was then selected and linked to a further chromeno-pyrazole moiety, obtaining a symmetrical (homo) bitopic ligand. The alkyl spacer was selected with a variable length, spanning from four to sixteen methylene units. Binding and functional studies showed the ability of these bivalent compounds to selectively activate CB2R, with linkers of 10-12 methylene units (**6** and **7**, Figure 5) giving the most promising results. Docking, molecular dynamics and site-directed mutagenesis studies evidenced the simultaneous binding of these compounds in the orthosteric site and in an exosite located at the entry channel connecting the orthosteric site and the lipid bilayer membrane.




R = 1,1-dimethylheptyl
n = 4-16

**6**, n = 10
**7**, n = 12

Ki CB1 > 40 μM
Ki CB2 = 300 nM

**Figure 5.** Bitopic ligands for CB2R

Notably, depending on the spacer structure and length, these bivalent compounds could be able not only to bind to orthosteric and exo- sites of the same receptor (dualsteric) [48], but also embroil two orthosteric sites of homo- or heterodimers. As such, they can be considered useful pharmacological tools for a better characterization of homo- and heterodimerization of CBRs and for the definition of the interactions between ECS and different receptor systems. A review dealing with the exploitation of bivalency in CBRs ligands development has been published in 2015 [13].

## 3. POLYPHARMACOLOGY INVOLVING CBRs

### *3.1 Cannabinoid receptor modulation in Alzheimer's Disease*

Despite the relevant ability of CBRs targeting compounds to counteract neuroinflammation, it is still unfortunately clear that the multifactorial nature of NDs makes it challenging to find an effective therapeutic strategy only based on ECS-targeting compounds. Undoubtedly, in such a complex framework, a multitarget-directed ligand (MTDL), a single molecule capable to interfere with multiple pathways involved in disease progression by engaging different selected targets, would represent a winning approach

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[52, 53]. In this respect, AD is certainly the most studied ND, probably considering the key role played by the inhibition of cholinesterases (ChEs) enzymes in the symptomatic relief during the development of the disease. Indeed, inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), leading to a boost in cholinergic activity, still remains the main mode of medical intervention, due to the well-known involvement of cholinergic system dysfunction in the progression of the disease [54]. These enzymes, responsible for acetylcholine hydrolysis and inactivation, act sequentially during disease development, being AChE mainly present in early AD stages and decreasing during disease evolution. On the contrary, BChE levels remain almost unchanged, thus becoming prevailing in late AD and able to counteract the decreased function of AChE. This peculiar behaviour makes AChE inhibitors suitable at the onset of the disease and BChE inhibitors more appropriate in later stages [55]. Moreover, BChE seems directly involved in cholinergic transmission, as it is overexpressed and co-localized in AD Aβ-enriched neuritic plaques and neurofibrillary tangles [56]. Both ChEs are then considered reliable and validated targets in AD. In this context, simultaneous inhibition of ChEs and CB2R agonist activity could synergistically act to restore cholinergic tone and reduce microglia-sustained neuroinflammation.

In general, a multitarget compound is obtained by hybridation or merging-based design strategies, in which two or more pharmacophoric fragments are joined to provide a new molecular entity (Designed multiple ligands, DMLs) [57]. Otherwise, compounds already reported as having a specific biological activity may retrospectively reveal a dual profile, being able to act on a different unexpected target. In this direction, some years ago, J. A. Paez and his group demonstrated the *in vitro* inhibitory activity of some aminoalkylindole-based cannabinoid agonists derived from JWH-015 and WIN 55,212-2 (Figure 6) on AChE or/and BChE enzymes, identifying for the first time a promising new class of dual inhibitors [58]. Starting from this finding, they reported a series of indazolyl ethers, designed by applying the bioisosterism principle, replacing the indole ring with an indazole core [59]. Moreover, for a better resembling with the aminoalkylindoles previously discovered and aiming at improving the binding to the CB2R, an aromatic ether was also introduced. A virtual library of indazole derivatives was then designed and a preliminary docking study on a theoretical model of CB2R developed by the group was performed.

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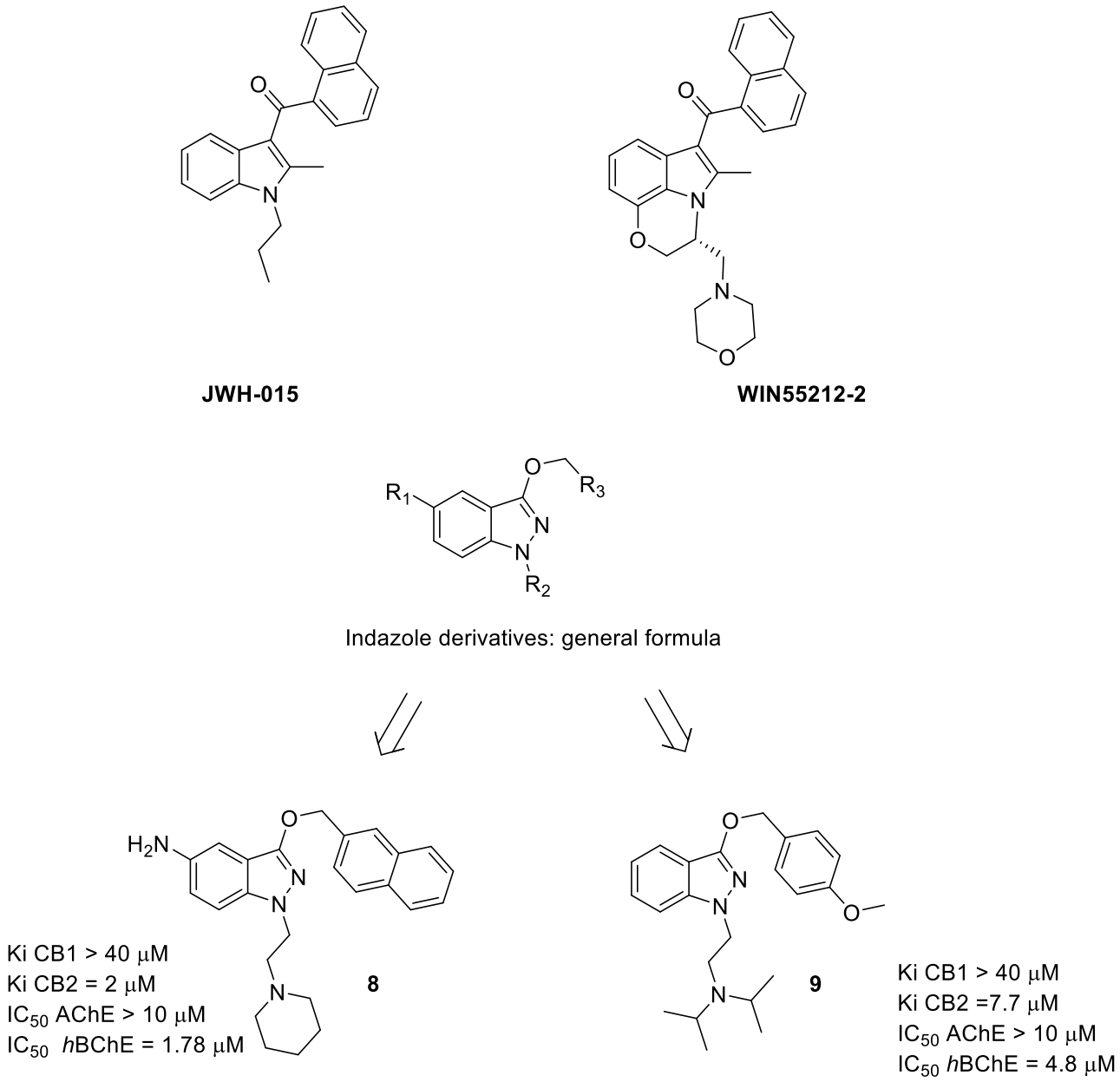


**Figure 6.** Development of indazole derivatives as MTDLs, starting from indole-based compounds.

The results suggested the introduction of further selected substituents in the *N*-1 position of the indazole core and the biological tests indicated for most compounds the ability to bind CBRs, some of them with a marked selectivity for CB2R, and to inhibit ChEs, in particular BChE. In a multitarget perspective, derivatives **8** and **9** (PGN-33) (Figure 6) emerged as the most promising, showing well-balanced CB2R agonist and BChE inhibitory activities and an appreciable antioxidant activity, of particular interest in treating age-related NDs.

In a follow-up study [60], the same research group identified PGN-33 as a valuable starting point for the hit-to-lead optimization of a dual BChE inhibitor/CB2R agonist, and attempted to also recruit the β secretase 1

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(BACE-1) enzyme, responsible for Aβ protein aberrant production through the β-site amyloid precursor protein cleavage, and then recognized as a valuable target for AD. Driven by a docking study, a *N*-1 carbonyl group was introduced to both improve the binding with CB2R and match one of the requirements of the BACE-1 pharmacophore model (Figure 7). This modification could probably lead to a different binding mode compared to PGN-33, with the H-bond acceptor carbonyl group able to establish new key interactions, both with CB2R and BChE. The new series of indazolylketones showed a multipotent profile, with compounds **10** and **11** (Figure 7) emerging as CB2R full agonists, able to inhibit BChE and, to a lesser extent, BACE-1 enzyme, also endowed with antioxidant and neuroprotective effects.

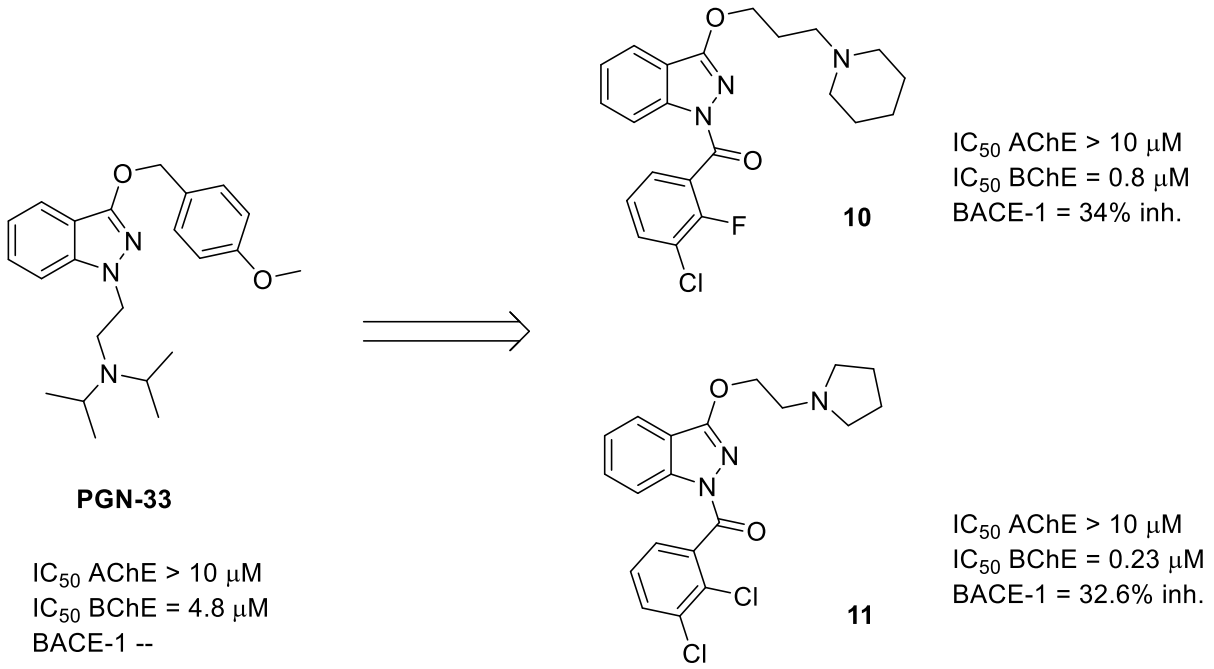


**Figure 7.** Development of indazolylketones as multifunctional compounds

The group of M. Decker has a long-lasting experience in cannabinoid research for NDs, and applying a previously developed pharmacophore model for BChE inhibitors to a benzimidazole-based CB2R agonist **12** (Figure 8) described by AstraZeneca and to the abovementioned dual acting indazole derivative PGN-33, planned a challenging "dual" SAR study, aiming at improving activity on both BChE and CB2R. [61] To validate this approach, the CB2R agonist **12** was assayed for BChE inhibition, showing an appreciable inhibitory activity and relevant selectivity over AChE, endorsing the starting hypothesis. Then, different fragments of the lead benzimidazole **12** were properly modified to better define the structural requirements for modulation of the selected targets, i.e. removing the diethylamide function to evaluate its possible role in interactions with BChE and CB2R, introducing a basic nitrogen atom into the alkyl chain bound to the benzimidazole core, modifying the benzyl substituent and finally substituting the benzimidazole with an indazole. In particular, the methylene unit of the benzyl moiety of **12** was replaced by an amino group, leading to a basic guanidine system more prone to establish H-bond interactions with both enzyme and receptor. A "second generation" library of 21 derivatives was then designed, synthesized, and tested on both targets, leading to the identification of benzimidazole- and 2-aminobenzimidazole-based selective BChE inhibitors active in the low micromolar range, also maintaining high selectivity over AChE. On the contrary, the indazole derivatives proved to be almost inactive on this target. The data also

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highlighted the pivotal role of the diethylamide moiety for BChE inhibition. In radioligand binding studies on *h*CB1 and *h*CB2 receptors, most of the compounds showed high selectivity for *h*CB2R, with affinity ranging from 127 nM to 26 μM, while remaining less active compared to **12**. Notably, the introduction of a-NH in place of the methylene in position 2 on the benzimidazole, leading to 2-aminobenzimidazoles, seems to be unfavourable on this target. From a multitarget perspective, the piperidine-substituted benzimidazole derivative **13** (Figure 8) appeared as a valuable, still unbalanced lead structure, showing a low micromolar inhibition of BChE ($IC_{50}$ = 2.3 μM) and high affinity for CB2R (Ki = 188.0 nM). To better rationalize the data, docking studies and molecular dynamics simulations were also performed on a subset of compounds for both targets. These data unveiled the differences in activity seen with the biological assays, indicating for the 2-aminobenzimidazole derivatives a different binding mode with respect to the lead compound. Indeed, comparing the computational studies for **14** (Figure 8) and **12**, differing only for a nitrogen in place of methylene in position 2, the impact of this N clearly appeared, leading for **14** to the loss of a π-π stacking in the case of CB2R, due to a different binding mode (reduced activity), and a gain of H-bond interactions for BChE (increased activity). The data on CB2R was also reinforced by the recovery in activity observed for **15** (Figure 8), in which the additional phenyl group could establish further interactions with the receptor, counteracting the lack of the 2-methylene group. Conversely, the presence of an aromatic system linked in 2-position of the 2-aminobenzimidazole or benzimidazole core seems to be required for dual activity.

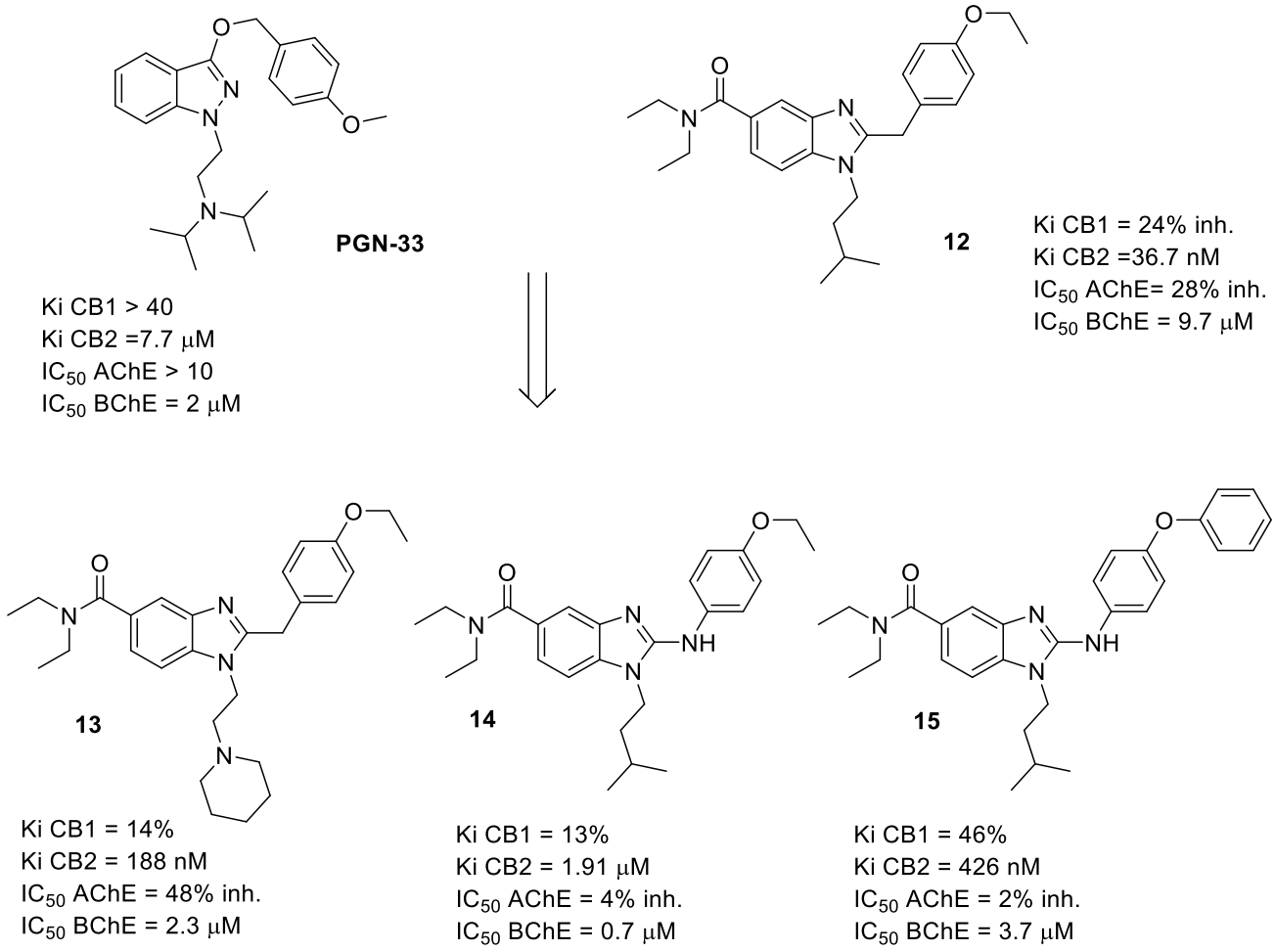


**Figure 8**. First and second generation of indole/indazole derivatives

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Without functional studies on CBRs activity, the therapeutic potential of these compounds cannot be properly evaluated, nevertheless, these findings could be useful to define a common chemical space for both targets, to be exploited and optimized in order to identify new lead compounds for NDs treatment. Indeed, in a follow-up study [62], the same group developed a third generation of related derivatives, aiming at obtaining well-balanced compounds on BChE and CB2R and, due to the structural similarity of the new compounds with μ opioid receptor ligands, designing out this undesired potential interaction. Moreover, the second and third generation compounds were evaluated in a functional assay to establish the effects on CB2R and, for the most active compound, an *in vivo* test was also performed in a murine pharmacological model of AD. Taking advantage of previously obtained results, the benzimidazole core was maintained and structural modifications were performed on the $N^1$-aminoalkyl chain, aiming at increasing the lipophilicity and potency on BChE, on the diethylamide in position 5, by modifying the amide function and introducing substituents with different electronic properties, and finally on the group in position 2. Different sets of compounds were then designed and synthesized, and some of them showed a promising low-micromolar balanced activity on both *h*BChE and CB2R. **18** and **19** (Figure 9) were the most active, and docking studies and molecular dynamics simulations were performed on these two derivatives, building a new homology model of *h*CB2R based on the crystal structure of *h*CB1R in complex with the agonist AM11542, published shortly before [63]. The compounds revealed a suitable fitting in the orthosteric binding pocket of the receptor, although with different orientations, maybe due to the differences in the chemical nature of the *N*-amido substituent. Interestingly, all derivatives proved to be CB2R agonists, and the structural requirements to avoid binding with the μ opioid receptor were defined. First, second, and third generation derivatives **16**, **17**, and **18** (Figure 9) were then tested in an *in vivo* murine AD model to evaluate their neuroprotective and procognitive effects, and the ability of a well-balanced dual-acting compound in enhancing cognition was demonstrated, showing **18** the most promising profile.

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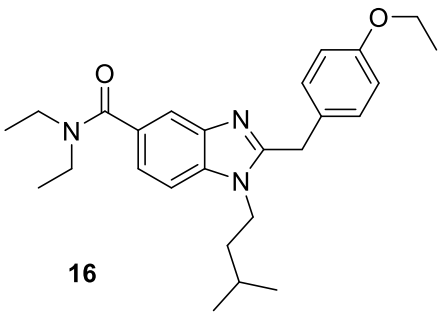
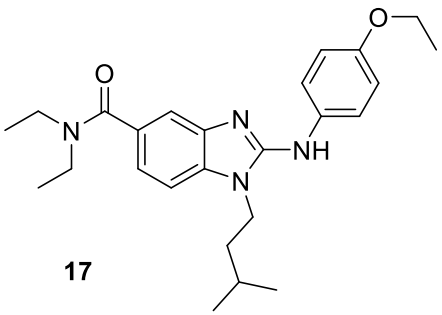


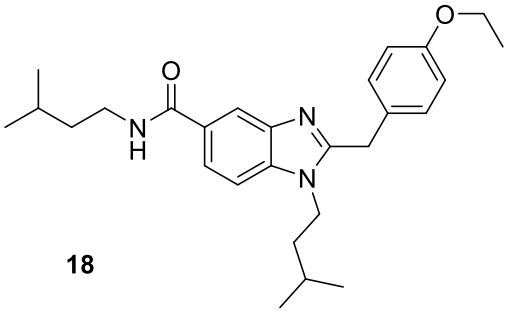
**16**

Ki CB1 = 24% inh.
Ki CB2 =36.7 nM
$IC_{50}$ AChE= 28% inh.
$IC_{50}$ eqBChE = 9.7 μM (inactive on hBChE)



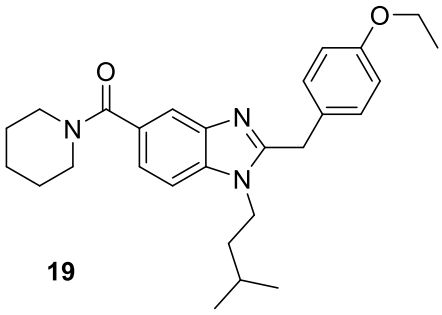
**17**

Ki CB1 = 13%
Ki CB2 = 1.91 μM
$IC_{50}$ AChE = 4% inh.
$IC_{50}$ eqBChE = 0.7 μM (inactive on hBChE)



**18**

Ki CB1 = 37% inh.
Ki CB2 = 763.6 nM
$IC_{50}$ AChE= 3% inh.
$IC_{50}$ hBChE = 1.6 μM



**19**

Ki CB1 = 17% inh.
Ki CB2 = 384.5 nM
$IC_{50}$ AChE= 8% inh.
$IC_{50}$ hBChE = 5.7 μM

**Figure 9.** Third generation of indole derivatives as multipotent compounds for AD

The same research group decided to exploit these studies by designing a hybrid molecule able to inhibit both ChEs and to act as an agonist of CB2R [64]. To this aim, tacrine, a well-known potent inhibitor of both AChE and BChE, was connected to the lead compound **16**, with proper selected linkers (Figure 10). Taking advantage of the previous SAR studies on **16**, the best positions for the insertion of the linker were identified in the *N*1 of the benzimidazole or the 5 amido group, leading to the synthesis of two subsets of compounds with different polymethylene chains. Once the optimal chain length was defined, the spacer was further functionalised by introducing a 2-poly-(ethylene glycol) (PEG) group to increase rigidity and polarity, in the *N*1 as well as in the 5-amido positions. Moreover, considering the hepatotoxicity induced by tacrine, a cystamine fragment was also linked at the amide position, aiming at increasing both

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neuroprotective and hepatoprotective properties (**17**, Figure 10) [65]. The results indicated a relevant ChEs inhibition, being some derivatives more active than tacrine itself. For these most significant compounds, a mixed inhibition of *h*AChE was also observed, leading to speculate on binding both to the central anionic site (CAS) and to the peripheric anionic site (PAS) of this enzyme. Due to the recognized role of PAS in *h*AChE-induced $A\beta_{42}$ fibrillization [66], this effect was also investigated, showing for **18** and **19** (Figure 10), but not for tacrine and **17**, the ability to substantially reduce the pro-aggregating effect of *h*AChE. On the contrary, while hybrids **17** and **18** strongly inhibited $A\beta_{42}$ self-aggregation, **19** was almost inactive. When tested for their binding to CBRs, all compounds showed a low micromolar affinity to *h*CB2R, and an agonist behaviour, evaluated for **18**, could be speculated for all these structurally related compounds. Finally, the effects of the hybrids on microglia activation and neuroprotection, as well as the hepatotoxicity, were assayed, and *in vivo* studies on an AD murine model were also performed, showing compounds **18** and **17** promising improvements of short- and long-term memory, low hepatotoxicity and ability to cross the BBB. Notably, the concept of exploiting tacrine in designing multitarget compounds has been widely explored [67, 68]. also aiming at obtaining a dual AChE inhibitor/CB1R antagonist/inverse agonist [69]. Indeed, the peculiar localization of CB1R in CNS still makes it an appealing target, despite the relevant weaknesses. The imidazole derivative **21** (Figure 11), developed by hybridization of tacrine and the 1,2 diarylimidazole **20** previously developed as a selective CB1R antagonist/inverse agonist, showed significant CB1 receptor affinity and selectivity towards CB2R, and inhibited AChE in the same potency range than tacrine.

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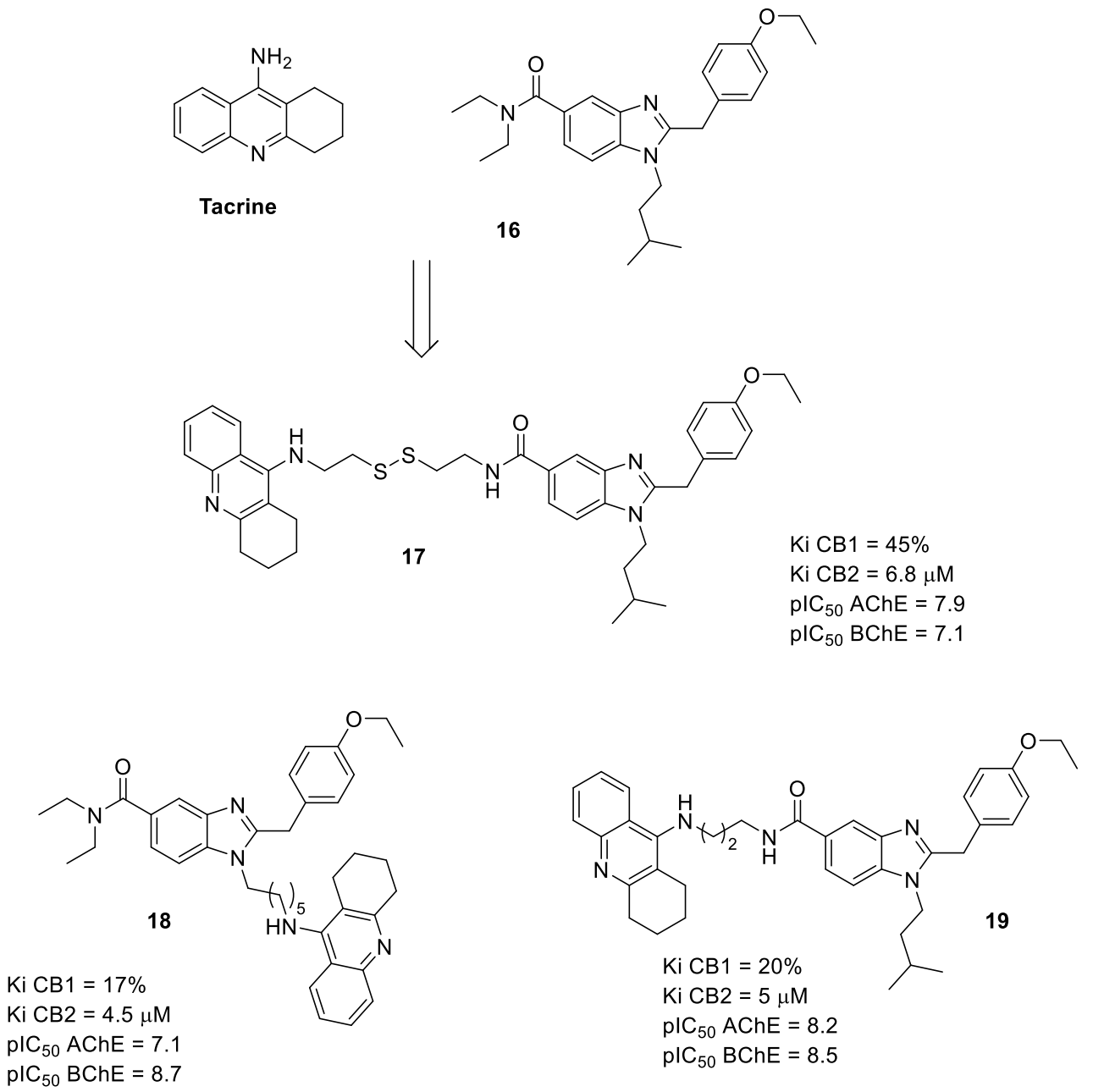


**Figure 10.** Hybrid tacrine-based derivatives targeting CB2R and ChEs

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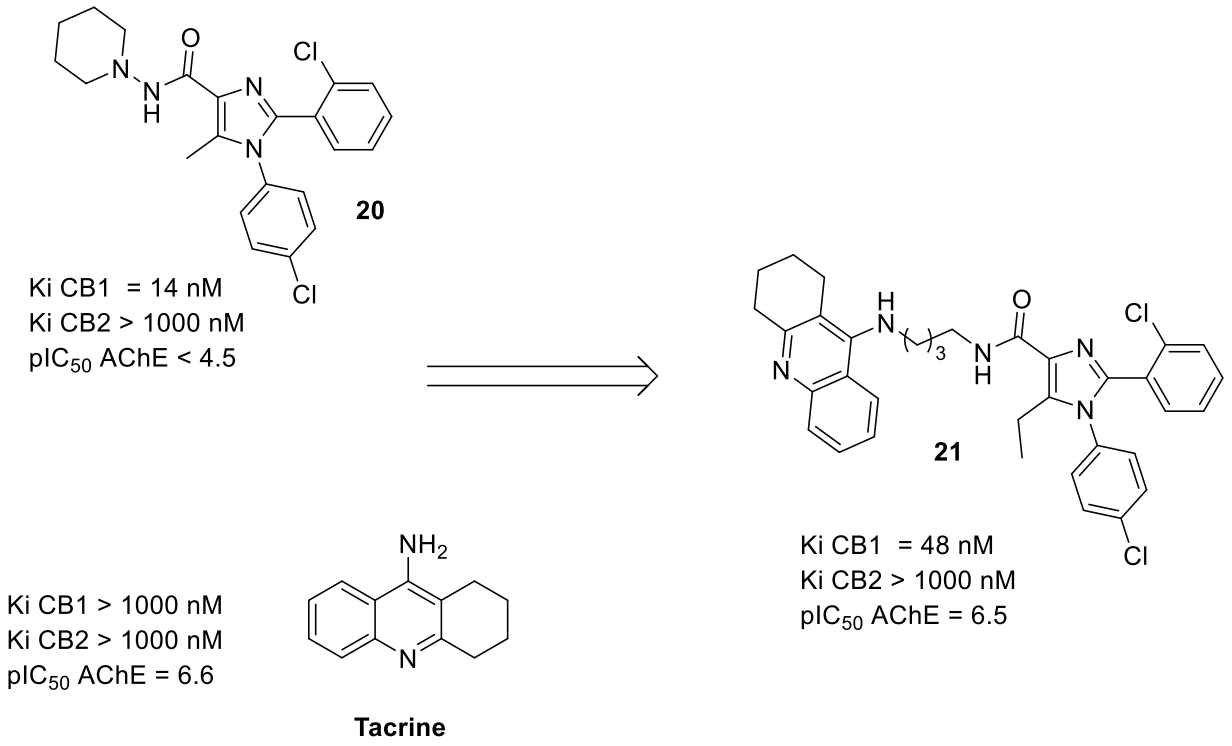


**Figure 11**. Tacrine-based molecules targeting AChE and CB1R.

Our research group has been dealing for a long time with the design and synthesis of multitarget compounds for treating AD, and in the last years our attention focused on ECS. In particular, the 2-arylbenzofuran hybrid molecule **22** (Figure 12), reported by us as a promising hit compound endowed with good inhibitory activity on AChE and BChE enzymes as well as a remarkable inhibition of Aβ aggregation and Aβ neurotoxicity [70], was considered a valuable template to be modified to broaden the biological profile by engaging CBRs. Indeed, the benzofuran core, a well-recognized privileged structure in medicinal chemistry, is also featured by LY320135, a high affinity antagonist/inverse agonist at CB1R [71]. Therefore, in continuing our studies on polyfunctional compounds in neurodegeneration, an extended library of 23 new 2-arylbenzofuran derivatives was designed [72] by modifying both the substituent in position 3 of the benzofuran scaffold and the spacer between this core and the *N*-methyl-*N*-benzylamine moiety, to determine its optimal length. Furthermore, the *N*-methyl-*N*-benzylamine heptyloxy side chain was moved from the *para* to the *meta* position of the phenyl ring. The compounds were tested to evaluate their activity on *h*AChE and *h*BChE and Aβ fibril formation. The most promising derivatives were then assayed for their ability to reduce Aβ neurotoxicity and to prevent Aβ peptide binding to the cell membrane and intracellular ROS formation in human neuronal SH-SY5Y cells. Besides, considering the peculiar benzofuran-based structure, the evaluation of the affinity for CB1 and CB2 receptors was also performed. Most of the compounds showed a better inhibition of BChE compared to AChE, some of them with an enhanced potency with respect to **22**, and some derivatives emerged as Aβ fibrils formation inhibitors and neuroprotectants, with a significant reduction of Aβ peptide-induced ROS formation. Regarding the affinity for CBRs, in this first study only a few structural features emerged, showing the lead compound **22** a good selectivity and a moderate affinity for human recombinant CB1R, while the introduction of an amino

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moiety on the benzoyl group attained a different effect depending on the position of the substituent: the amino group in the *para* position appeared to modulate affinity, leading to an increase in selectivity for CB2R, as in compound **23** (Figure 12). Moreover, moving the heptyloxy chain from the *para* to the *meta* position proved to be detrimental for activity on CBRs.

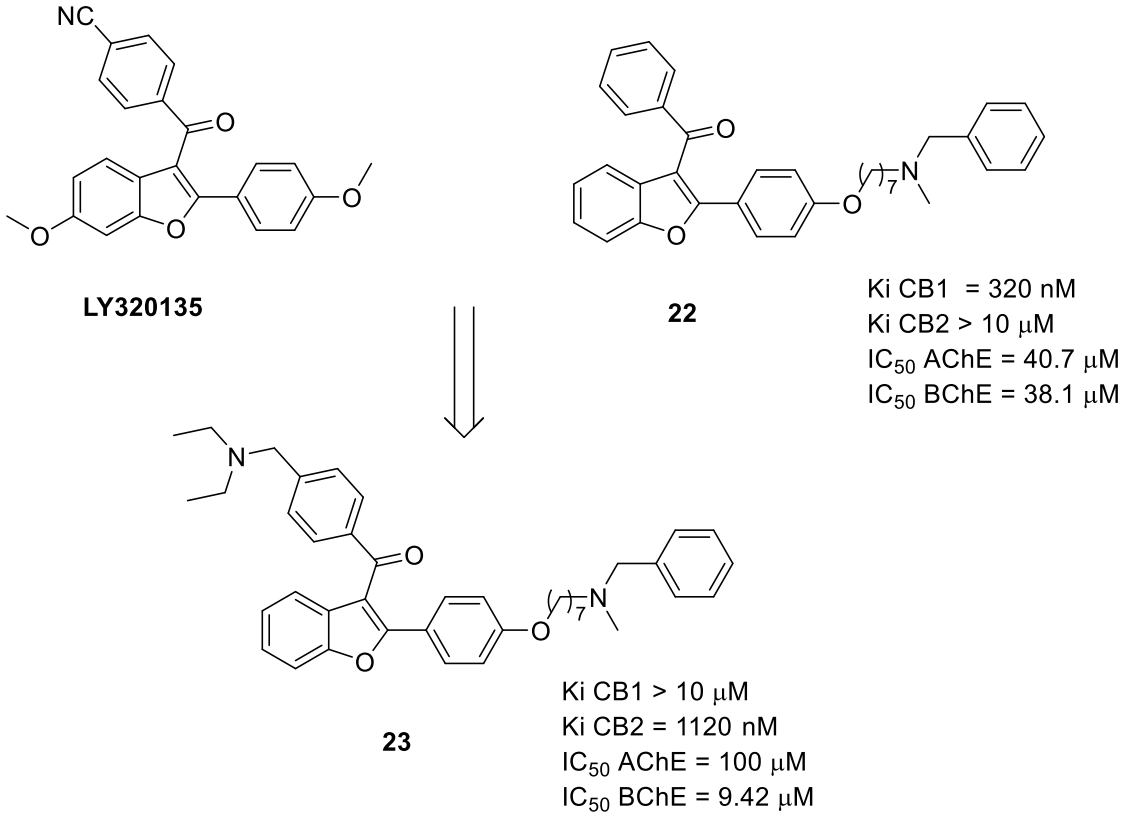


**Figure 12.** SAR study on benzofuran derivatives.

In a follow-up study, new modifications were performed on **22**, aiming at obtaining new balanced multipotent compounds and expanding the SARs [73]. A first series was designed, in which the side chain was moved from the A to the B ring, while in the second series the linker chain was removed with mono-or di-alkylated amines directly linked to the B ring (Figure 13). These modifications allowed achieving promising results both on ChEs and CBRs. In particular, most of the compounds showed selective BChE inhibition, with increased potency with respect to **22**, and several potent CBRs ligands were also obtained, endowed with peculiar selectivity and mainly an inverse agonist behaviour. In the first series, affinity for the two CBRs seemed to depend on the length of the linker, while in the second series potent ligands for these receptors were discovered, being compound **23** (Figure 13) endowed with significant affinity for both receptors, with a preference of about 40 times for CB2R, and its *N*-methyl derivative **24** (Figure 13) showing the highest CB2R binding affinity. Moreover, compound **23** proved to substantially inhibit BChE and to exert a neuroprotective activity against Aβ oligomers, combining anti-inflammatory and neuroprotective effects. In addition, since proinflammatory M1 microglia predominate in the advanced stage of AD and aggravate disease progression, compound **23** could be effective in halting lesion development by interfering with the activation of M1. On the other hand, **24** emerged as a potent CB2R inverse agonist with promising immunomodulatory properties and could be considered as a new probe for a better understanding of the role of CB2 receptors in immunomodulation.

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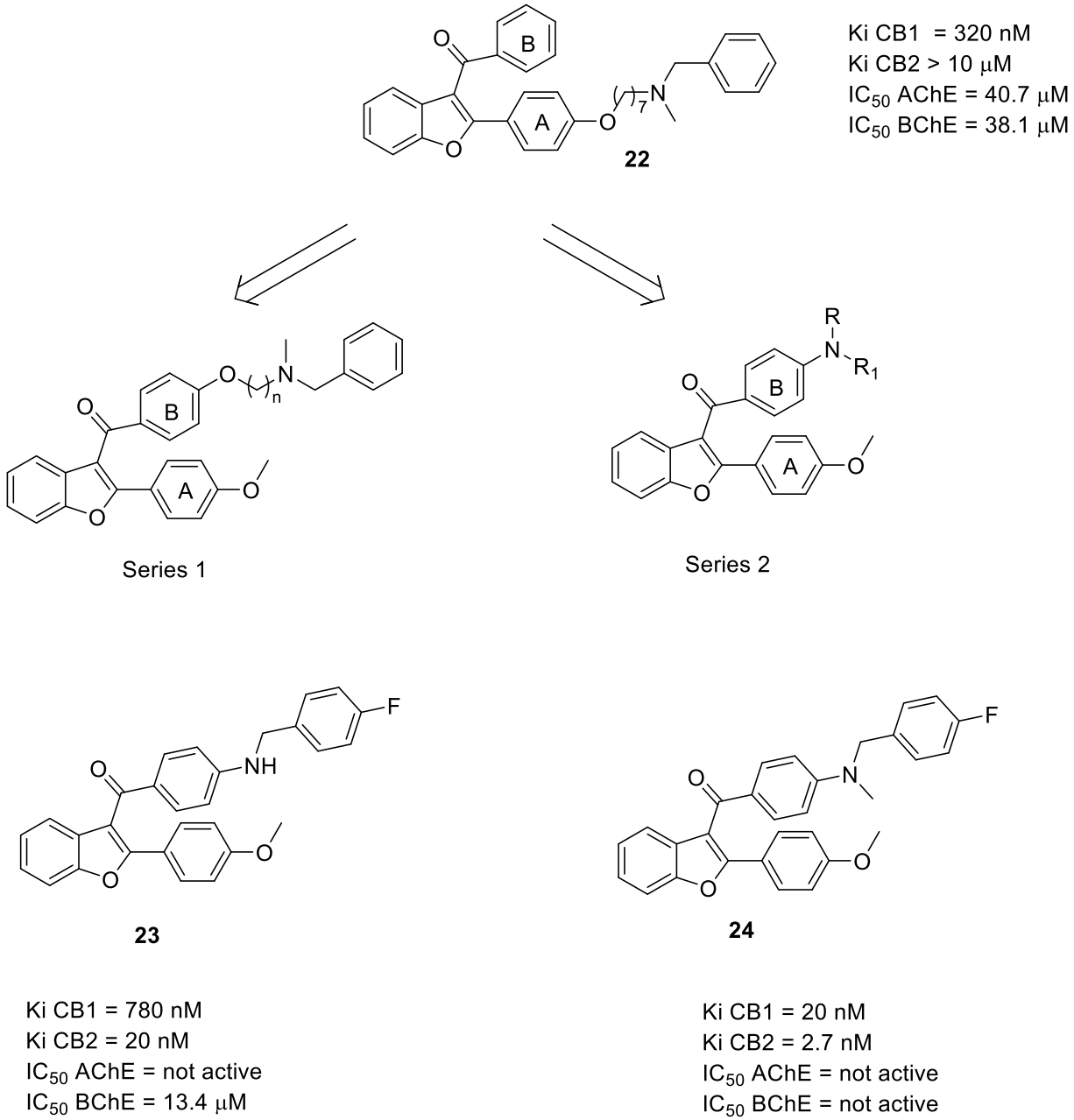


**Figure 13**. Further development of benzofuran derivatives.

*3.2 Cannabinoid receptor modulation in Multiple Sclerosis*

MS is a CNS chronic autoimmune disease responsible for a progressive disability condition, particularly devastating for young adults. As in most NDs, its aetiology has not been clarified yet, but several lines of evidence lead to the conviction that MS development results from an aberrant autoimmune response, in which the immune system attacks and disrupts the myelin sheath surrounding neurons, hampering the transmission between brain and body [74]. Disease progression is depending on both progressive demyelination and failure to remyelinate. Therefore, MS can cause permanent damage of the nerve fibres,

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leading to the main clinical symptoms and neurological deficits: spasticity and the resulting complications such as pain, sleep disorders, and depression [75]. In MS, a detrimental combination of inflammatory and neurodegenerative processes, variously prevalent in the different stages of the disease, can be noticed, and pharmacological treatments with immunomodulators and centrally acting muscle relaxants, such as baclofen, benzodiazepines and dantrolene, represent the main line of intervention. However, an effective therapeutic regimen able to manage such a complex picture is far from being found, considering that currently used drugs are associated with significant side effects and a substantial number of patients are unresponsive [76].

To date, no DMLs are reported for MS treatment, however, some phytocannabinoids, namely THC and CBD, currently used in clinical practice for MS, proved to interfere with multiple mechanisms underpinning the disease, being able to be considered multitarget compounds. These galenic standardized formulations are usually dispensed as oral infusion or vaporization [77]. Sativex, a mixture of two extracts of THC and CBD, administered as an oromucosal spray, is a further therapeutic option for unresponsive spasticity in MS patients. It proved to be noninvasive and effective in treating spasticity, in particular as an adjunctive therapy, showed a good safety profile and appeared well tolerated, and was approved in Italy in 2013 [78].

In 2017, Annunziata *et al*. [79] reported a deep pharmacological study on **COR167** (Figure 14), a synthetic CB2R selective agonist previously discovered [80]. They concluded that CB2R selective stimulation plays a remarkable role in counteracting neuroinflammation and that the *ex vivo* immunomodulatory effects demonstrated in the study coupled with the potent anti-nociceptive properties previously reported for the compound make COR167 worthy of further investigation.

In recent years, semisynthetic cannabinoids were designed aiming at improving both activity and druggability. **VCE-004.8** (Figure 14), an aminoquinone derivative of CBD, is a dual PPARγ and CB2R targeting compound [81] able to modulate neuroinflammation. A following study demonstrated the ability of this compound to activate the hypoxia-inducible factor (HIF), which could in turn activate different cellular pathways involved in neuroprotection and axonal regeneration [82].

In 2016, Morales *et al*. accomplished a molecular modelling-guided SAR study on their previously reported chromeno-pyrazole derivatives acting as CB1R agonists, aiming at shifting on CB2R, to obtain nonpsychoactive compounds for MS treatment [83]. A series of 36 new derivatives were synthesized with a wide range of structural modifications, and CBRs binding assays coupled to functional studies were performed. Low-nanomolar chromeno-pyrazole-based CB2R ligands were found, among which **25** emerged as the most potent (Figure 14), but the most promising compound, also endowed with high selectivity with respect to CB1R, was their previously described neuroprotective agent **PM226** (Figure 14). This compound was then tested in the acute inflammatory phase of TMEV-IDD, an animal model of primary progressive MS, showing a relevant reduction in neuroinflammation by decreasing microglial activation.

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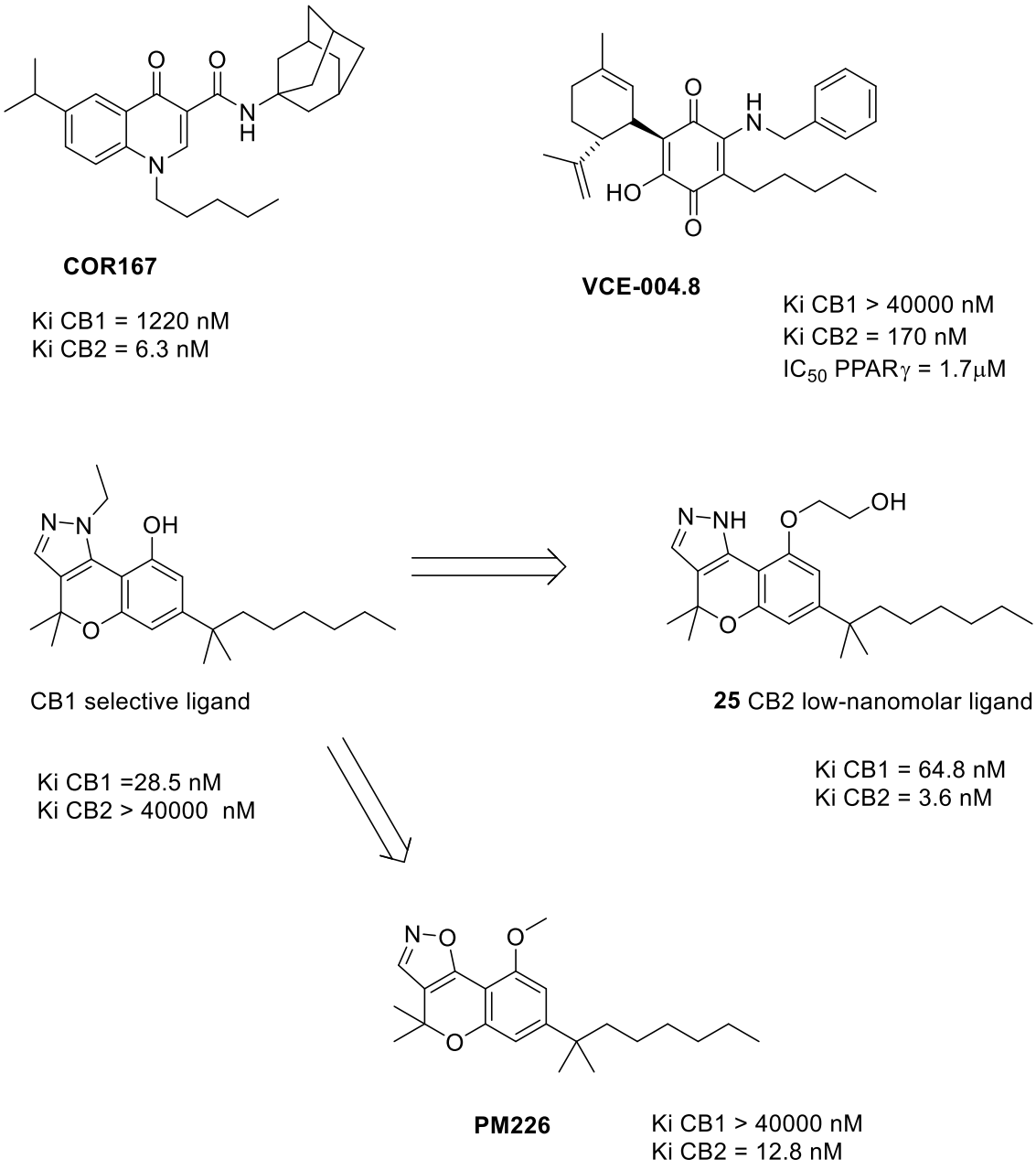


**Figure 14.** CBRs ligands for MS

*3.3 Cannabinoid receptor modulation in Parkinson disease*

The involvement of CBRs in PD has been widely considered in these last years, to conceive new therapeutic options aiming at alleviating specific motor and nonmotor symptoms and halting disease progression [31, 33]. In the brain of PD patients, differential expression of CB2R can be observed, suggesting its prime role in PD pathogenesis compared to CB1R [32]. However, a shortfall of clinical data on the application of CBs in PD patients can be noticed, even if preclinical studies proved that cannabinoids targeting CB1R were able to attenuate parkinsonian symptoms, namely bradykinesia and immobility, while compounds acting on CB2R and GPR55 reduced neuroinflammation, one of the triggering features in PD development [35]. Moreover, CBRs independent effects, mainly a decreased oxidative injury, could also help in counteracting the

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worsening of the disease [34]. To date, levodopa still remains the chief symptomatic therapy of PD, together with dopamine agonists, monoamino oxidase B (MAO-B) inhibitors, and anticholinergic drugs, but the benefits of the therapy decrease over time. The ever-increasing interest in finding alternative non-dopaminergic drugs is well documented by the numerous reviews published in these last years dealing with the possible employment of CBRs interacting compounds to relief PD symptomatology [31, 32, 35, 36, 84].

Despite these efforts, purposely designed multitarget compounds involving ECS targets have not been reported to date, but a few compounds have been extensively studied and show a multipotent profile, among which the eCB transporter inhibitor/vanilloid agonist AM404 [85] and the CB1R/CB2R agonist CP55,940 [31] (Figure15). These compounds are also endowed with antioxidant properties, likely due to a CBRs independent mechanism.

Recently, VCE-003.2 (Figure 15), a CBG quinone derivative, has been evaluated for its neuroprotective properties in murine inflammatory models of PD [86]. This potential is related to its activity at the PPAR-γ receptor, rather than a direct binding to CBRs. VCE-003.2 effectively worked as an anti-inflammatory and neuroprotective agent in different *in vivo* PD models. *In vitro* studies further assessed the key role of PPAR-γ receptors for these effects, supporting the idea that a multitarget compound obtained by merging a pleiotropic cannabinoid derivative targeting PPAR-γ receptors and other relevant targets involved in PD development, may lead to the discovery of a disease-modifying agent in PD.

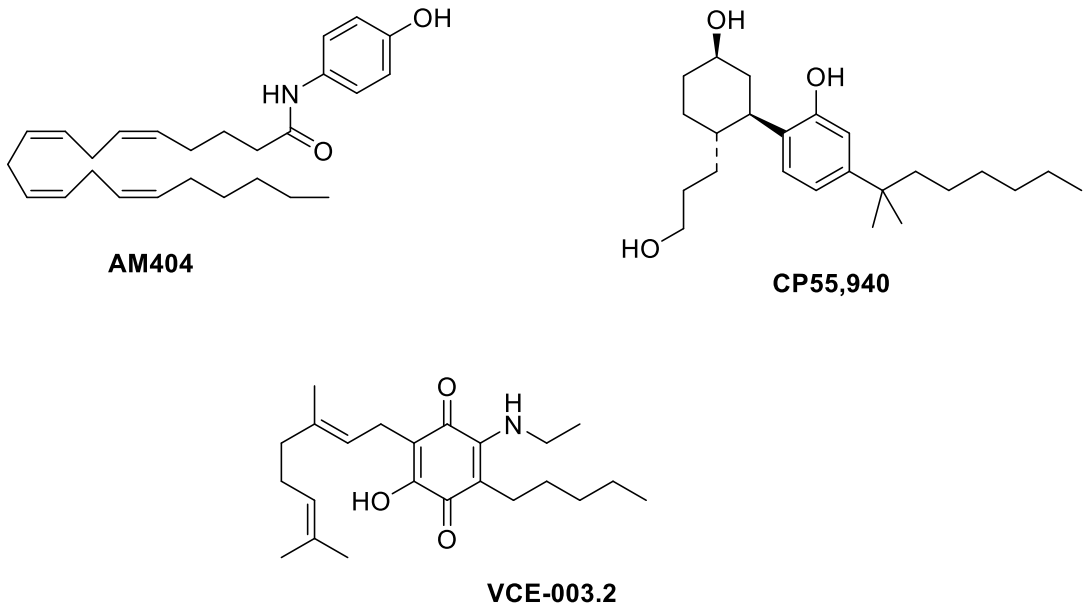


**Figure15**. ECS modulators for PD

*3.4 Cannabinoid receptor modulation in Huntington disease.*

HD is a genetic ND caused by the expression of a mutant huntingtin protein with expanded glutamine repeats in the *N*-terminal portion of the protein, that forms intranuclear inclusions also involving other molecules and finally induces the transcriptional dysregulation of numerous key genes together with striatal atrophy and death of medium spiny neurons. The main symptoms in HD are abnormal involuntary movements (chorea), related to the degeneration of the striatum, and cognitive decline, linked to cortical

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deterioration. Therapeutic treatments for HD patients are very limited and preventing or slowing the progression of this devastating disease is still an unfulfilled goal [36, 37].

Several studies underpinned the relevance of CBRs as potential targets for neuroprotective therapy of HD. In particular, microglial CB2Rs elicited neuroprotective effects by reducing neuroinflammation, striatal neuronal loss, and hence motor symptoms [35]. Although the cellular localization of CB2Rs is still unclear, it could be presumed that targeting CB2R may constitute an effective therapeutic option for HD treatment [37].

Interestingly, the PPARγ activator VCE-003.2, also evaluated in PD model, showed neuroprotective activity against mutant huntingtin-induced damage and improved neurogenesis [87-88].

## 4. POLYPHARMACOLOGY INVOLVING FAAH

Endogenous neuromodulatory molecules are commonly coupled to specific metabolic enzymes in order to achieve rapid signal inactivation. In 1996, Cravatt *et al.* showed that oleamide hydrolase could also convert AEA to arachidonic acid, indicating that this enzyme could inactivate a large family of bioactive signaling molecules [89] Therefore, this hydrolase was named FAAH, fatty-acid amide hydrolase, in recognition of the plurality of fatty-acid amides that the enzyme can accept as substrates. FAAH is an integral membrane enzyme belonging to the superfamily of serine hydrolases, it is widely distributed throughout the body and inactivates eCBs [90]. The catalytic mechanism of FAAH is unique among mammalian enzymes, since it involves a catalytic triad consisting of two serine (Ser217 and Ser241) and one lysine (Lys142) residues [91]. Genetic or chemical inactivation of FAAH enzyme results in increased neuronal transmission leading to analgesic, anti-inflammatory, anxiolytic, and antidepressant effects [92], without producing the undesirable side effects observed with CB1R agonists [93]. Thus, FAAH emerged as a promising target for several disorders related to the peripheral and central nervous systems.

The crystal structure of rFAAH was established and characterized for the first time in 2002 by Bracey and co-workers [94], and since then both rational drug discovery and computer-aided drug design by several research groups have led to the synthesis of numerous inhibitors with different scaffolds. The results provide new evidence for the prominence of ECS in pain modulation and reinforce the proposed role of FAAH as a target for analgesic drug development. One of the most studied inhibitors is URB567 (Figure 16) [95], which has reached clinical trials. Recent studies reported that FAAH inhibition through URB597 decreased neuroinflammation and improved hippocampal-dependent memory [96], suggesting its potential usefulness for the treatment of AD. Furthermore, other researchers demonstrated that modulation of ECS through URB597-mediated FAAH inhibition was beneficial for the motor symptoms of PD [97].

Another research group developed a new series of highly potent and selective carbamate FAAH inhibitors (Figure 16, general structure and representative compound **26**) and reported their evaluation for neuroprotection. Some inhibitors displayed protection against the synaptic deterioration produced by kainic acid-induced excitotoxicity, suggesting that they may have future therapeutic potential for various disorders [98].

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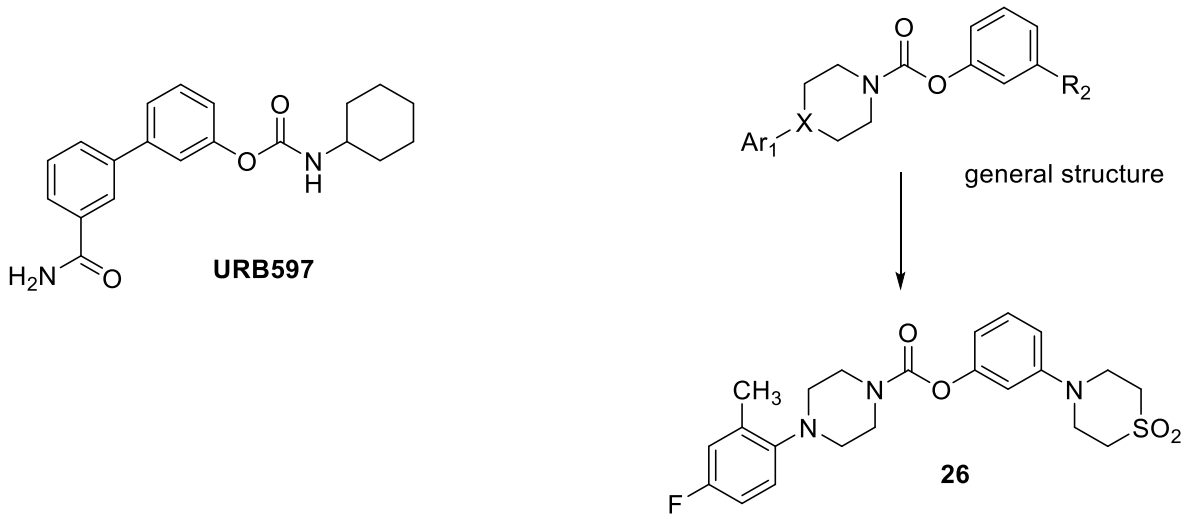


**Figure 16**. FAAH inhibitors with neuroprotective activity.

Several reviews report the progresses achieved in recent years in FAAH inhibitors design [99, 100], while this chapter will focus on FAAH inhibitors endowed with multitarget activity to be used for treating neurodegeneration.

*4.1 FAAH/AChE/BChE inhibitors*

In AD, an increased activity of FAAH was selectively demonstrated in regions of Aβ-enriched neuritic plaques [101]. On the other hand, early inhibition of eCBs inactivation was found to reduce Aβ-induced gliosis, neuronal death, and memory retention loss [102]. Taking into account the above-mentioned issues, our research group, engaged for many years in the development of ChEI carbamates, decided to test the in-house carbamate library on this new target. Starting from the data obtained on FAAH, it was possible to carry out the substantial SAR study that allowed us to design four new carbamates, two with a coumarin nucleus and two with an azaxantone one, both substituted with an appropriate bulky group on the carbamate to maintain the key features required for ChE inhibition, with the aim of obtaining MTDLs compounds [103]. Derivatives **27** and **28** emerged (Figure 17) as the first dual cholinesterase-FAAH inhibitors. This dual activity could lead to a potentially more effective counteraction of AD progression, because they would allow regulation of both ACh and eCB signaling and improve neuronal transmission and/or reduce neuroinflammation.

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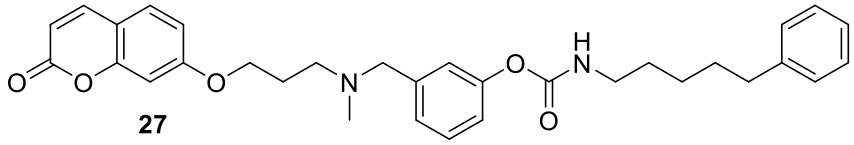
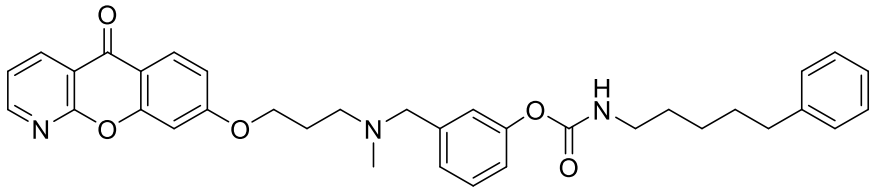


**27**

FAAH: $IC_{50}$= 50 nM
AChE: $IC_{50}$= 75 nM
BChE: $IC_{50}$= 1.6 nM

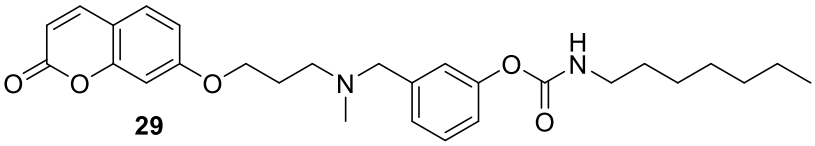


**28**

FAAH: $IC_{50}$= 40 nM
AChE: $IC_{50}$= 89 nM
BChE: $IC_{50}$= 1.7 nM

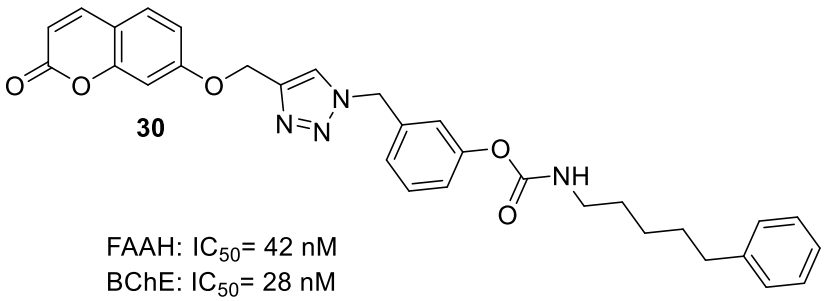
**Figure 17**. The first dual ChE/FAAH inhibitors.

Our research group has continued this study over the years to increase the potency of the compounds and obtain well-balanced activities on the selected targets, namely FAAH, AChE, and BChE. Therefore, appropriately designed modifications were performed on the lead molecule **27**, in order to explore the chemical space of these inhibitors and to determine the structural features required for obtaining higher potencies. Various carbamic functions were inserted in selected oxygenated heterocycles, related to natural products, with spacer chains of different lengths or including a triazole ring, to evaluate the impact of a lower flexibility and the role of the basic center on biological activities. Among the two series of synthesized compounds, some derivatives proved to be extremely potent on a single target, while compounds **29** and **30** were identified as effective dual FAAH/ChE inhibitors (Figure 18) [104].



**29**

FAAH: $IC_{50}$= 28.5 nM
AChE: $IC_{50}$= 37.4 nM
BChE: $IC_{50}$= 1.36 nM



**30**

FAAH: $IC_{50}$= 42 nM
BChE: $IC_{50}$= 28 nM

**Figure 18**. New dual ChE/FAAH inhibitors.

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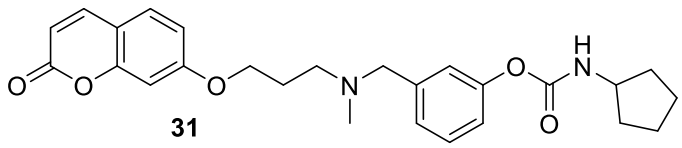
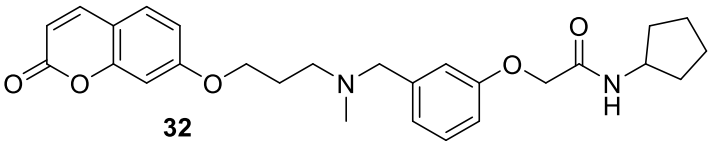
Despite the fact that ChEs and FAAH greatly differ in both tridimensional structure and binding site topology, **29** showed an improved inhibitory activity on all selected targets, while **30** was identified as a dual inhibitor, with nanomolar and well-balanced activities on BChE/FAAH. Hence, considering that ChEs levels vary during disease progression, with key implications in the selection of AChE or BChE as main target for treatment, **30** could represent a promising drug candidate for tackling moderate forms of AD.

Recently, in a following paper, additional insights on the structural requirements for dual activity were attained, further expanding the understanding on dual FAAH/ChE inhibitors potential. In particular, appropriately designed modifications were performed on our lead compound **29**, focusing on the carbamate group: this portion was modified by enclosing the heptyl chain of **29** in a 5- or 6-membered ring or by introducing a *N*-naphthyl substituent [105]. These modifications, aimed at maintaining or reducing the considerable lipophilicity of the parent compound, could improve the fitting into the pocket of the target enzymes. Notably, the cyclohexyl carbamate is also present in the selective FAAH inhibitor URB597 (Figure 16). Moreover, the carbamate was replaced with an amide, widely reported as a valuable group in medicinal chemistry, due to its ability to form crucial hydrogen bond interactions. This substitution aimed at elucidating the role of the carbamate function in enzyme blockade and evaluating the feasibility of attaining reversible inhibitors of ChEs and FAAH.

Furthermore, the new derivatives were also evaluated on a panel of selected targets embroiled in AD.




**31**




**32**

**Figure 19**. New multitarget agents.

In the carbamate series, **31** (Figure 19) was endowed with promising inhibitory activities on AChE, BChE, FAAH, and $A\beta_{42}$ self-aggregation, combined with lower QPLogPo/w, which leads to higher predicted solubility and potential improved *in vivo* activity, and calculated brain to blood ratio better than the reference *n*-heptyl compound. The potencies related to the single activity of this compound appear moderate, but, remarkably, all selected targets are involved in the pathogenesis and progression of the same disease and, from a multitarget point of view, this biological profile acquires a pivotal relevance. Indeed, **31** may counteract AD by simultaneously modulating different networked pathways involved in disease development.

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Taking advantage of the widespread occurrence of amide function in potent CB ligands, the ability of the new compounds of the amide series to bind these receptors was also evaluated. Amide **32** (Figure 19) showed an encouraging multitarget profile, inhibiting AChE, BChE, and $A\beta_{42}$ self-aggregation, and proving to be a potential CBRs ligand.

Tripathi *et al.* focused on an oxindole scaffold, an important and versatile structural motif found in an array of natural products and pharmacological agents [106]. The oxindole scaffold has evidenced a significant potential for a wide range of biological applications, and several 3-hydroxyoxindole analogs are in the preclinical evaluation stage. Taking this into account, the authors screened a library of 3-hydroxy-3-substituted oxindole analogs of isatin with a short, less flexible acetyl linker (general structure in Figure 20) with the purpose of inhibiting FAAH and ChEs. The study revealed some highly potent inhibitors. In particular, **33** (Figure 20) was the most promising compound, showing a balanced multifunctional profile on FAAH (competitive and reversible), AChE (mixed-type and reversible) and BChE (mixed-type and reversible) inhibition.

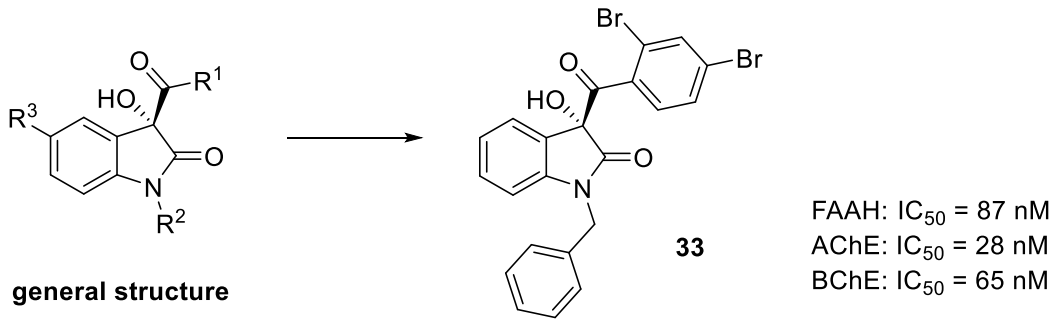


**Figure 20**. General structure 3-hydroxy-3-substituted oxindole analogs and the most potent inhibitor of FAAH, AChE, and BChE.

Compound **33** also exhibited an antioxidant potential, surpassing that of ascorbic acid, and was non-neurotoxic. *In silico* molecular and ADMET properties anticipated the druggability of this compound for oral use.

Hadizadeh and co-workers reported new carbamates designed as inhibitors of FAAH and ChEs inspired by the structure of the native substrates (AEA and ACh), the tridimensional structure of active sites and the SARs of the well-known inhibitors of these enzymes, according to the four substructures (A-D) shown in Figure 21 [107]. From this study, compound **34**, a heptyl carbamate analog possessing a 2-oxo-1,2-dihydroquinolin ring and an aminomethyl phenyl linker, emerged as the most potent inhibitor (micromolar range) for the three main targets.

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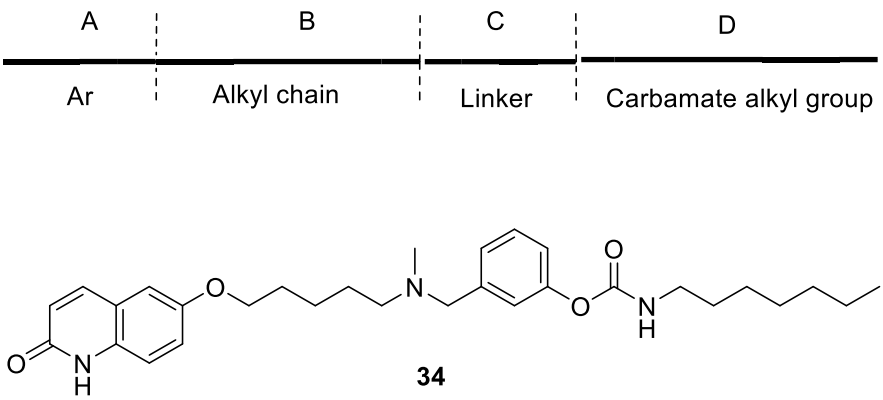


FAAH: $IC_{50}$= 0.83 μM
AChE: $IC_{50}$= 0.39 μM
BChE: $IC_{50}$= 1.8 μM

**Figure 21**. Four substructures A (Aromatic rings), B (Alkyl chain), C (Linker), and D (Carbamate alkyl group) and the most potent inhibitor of FAAH and ChEs.

In addition, **34** exhibited memory improvement in hyoscine-induced impairment in the Morris water maze test. Molecular docking studies showed that **34** could interact with the key residues of the enzymes binding sites and has the ability to form a stable ligand–receptor complex with the enzymes active site.

Lehr and co-workers initially developed a series of phenyl 4-[(indol-1-yl)alkyl]piperidine carbamates as FAAH inhibitors with general structure A (Figure 22). SAR studies revealed that variation of the length of the alkyl spacer connecting the indole and piperidine heterocycles, as well as opening of the piperidine ring, significantly affected activity. Indeed, compounds with general structure B were more potent inhibitors, unfortunately endowed with a significantly lower metabolic stability compared to the corresponding carbamates with general structure A [108]. Afterwards, further SAR studies on this type of compounds were performed with the aim of obtaining MTDLs [109]. In particular, a fluorine atom was introduced in position 6 of the heterocycle of B and the carbamate phenyl residue was replaced by differently substituted phenyl and heteroaryl moieties. Potent selective and chemically / metabolically stable FAAH inhibitors were obtained as a result of these studies, and from a multitarget point of view, compound **35** (Figure 22) emerged for its well-balanced inhibition of both FAAH and BChE and a reasonable hydrolytic stability.

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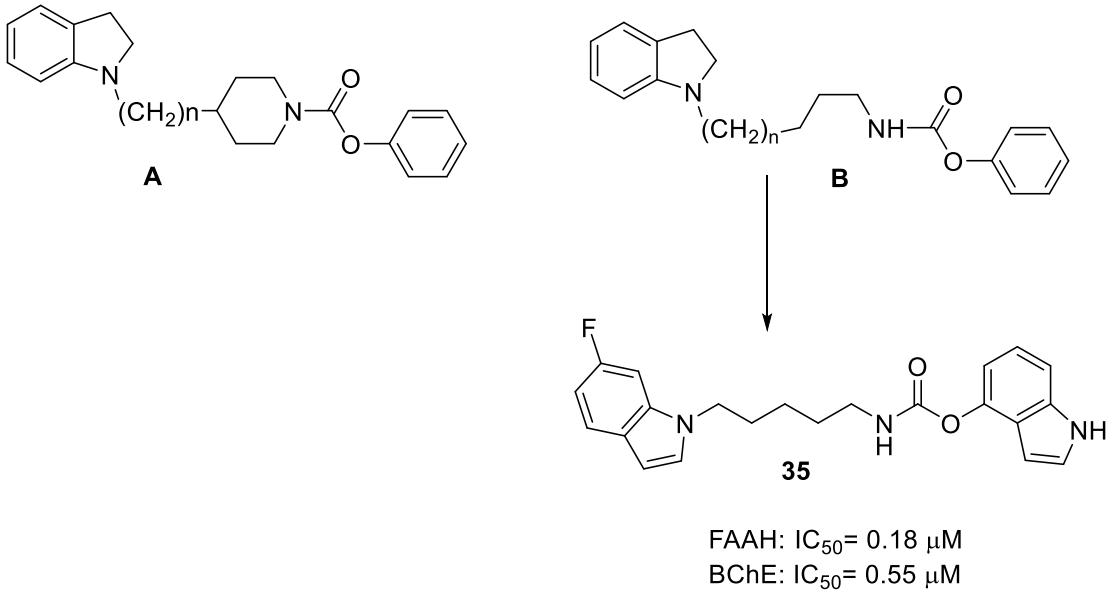


**Figure 22**. Indole-based phenyl carbamates and the most potent inhibitor of FAAH and BChE.

*4.2 FAAH inhibitors/CB ligands*

Manera and co-workers exploited their 1,2-dihydro-2-oxo-pyridine-3-carboxamide derivatives previously reported as CBRs ligands in a multifunctional approach, targeting both CBRs and FAAH. Indeed, it is reasonable to assume that the simultaneous modulation of more targets within the ECS may offer a safer and more effective drug strategy for addressing the complexity of this system. In 2018, they reported a new series of 4-substituted and 4,5-disubstituted 1,2-dihydro-2-oxo-pyridine-3-carboxamide derivatives endowed with a broad spectrum of affinities and functional activities on different components of the ECS. In this study, the scaffold was modified by insertion of a methyl group or an aryl moiety at the 4-position. Moreover, the new compounds are characterized by the presence of a *p*-fluorobenzyl moiety at the N-1 position of the 1,2-dihydro-2-oxo-pyridine ring and a *N*-cycloheptyl carboxamide group at the 3-position that were chosen on the basis of the best results obtained with their previous series. Compound **36** (Figure 23) showed a significant activity towards FAAH, high affinity for CB1R (partial agonist) and CB2R (inverse agonist), and a moderate inhibition of the 2-AG degrading enzyme α,β-hydrolase domain-12 (ABHD12) [110].

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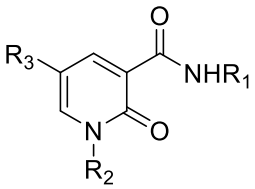
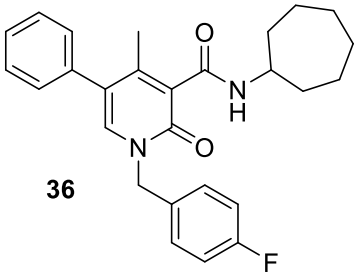
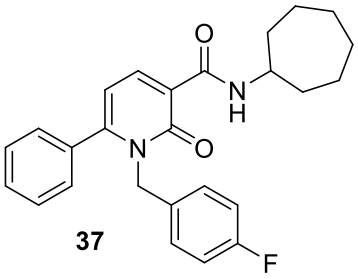



general formula



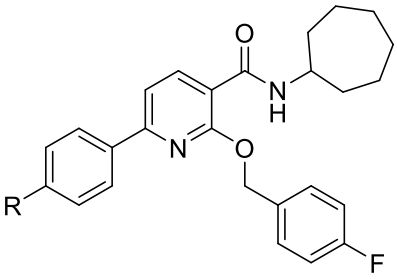

**36**

$IC_{50}$ FAAH = 70 nM
Ki CB1 = 23.1 nM
Ki CB2 = 6.9 nM
$IC_{50}$ ABHD12 = 2.5 μM




**37**

$IC_{50}$ FAAH = 2.9 μM
Ki CB1 = 304 nM
Ki CB2 = 3.1 nM
$IC_{50}$ ABHD12 = --
$IC_{50}$ AEA uptake = 62 nM




R = H, **38**: $IC_{50}$ FAAH = 70 nM
$IC_{50}$ AEA uptake = 76 nM

R = $OCH_3$, **39**: $IC_{50}$ FAAH = 64 nM
$IC_{50}$ AEA uptake = 96 nM

**Figure 23**. 1,2-dihydro-2-oxopyridine-3-carboxamide derivatives.

A further optimization study on these derivatives led to the discovery of the multifunctional compound **37** (Figure 23), showing moderate FAAH inhibition together with appreciable affinity for CB1R (with a partial agonist behaviour), high affinity and inverse agonist activity for CB2R. Remarkably, this compound proved to substantially reduce AEA uptake [111]. On the other hand, the corresponding 2-alkoxypyridine analogues **38** and **39** exhibited significant inhibitor activity on FAAH with nanomolar potency, without affecting the major 2-AG degrading enzyme MAGL, and on AEA uptake without significantly binding to both cannabinoid receptor subtypes. Moreover, molecular docking analysis was carried out on the three-dimensional structures of both CBRs and FAAH to rationalize the SARs of this series of compounds.

## 5. magl as a PROMISING target in polypharmacology

MAGL is a further important member of the serine hydrolase superfamily and plays a crucial role in the hydrolysis of monoacylglycerols into glycerol and fatty acids. In detail, it is confirmed as the primary hydrolytic enzyme for the degradation of 2-AG into arachidonic acid (AA), the precursor of proinflammatory prostanoids, and glycerol (Figure. 24). It is responsible for approximately 85% of 2-AG hydrolysis, while

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other two minor enzymes, ABHD6 and ABHD12, were shown to mediate around 9% and 4% of 2-AG degradation, respectively [112].

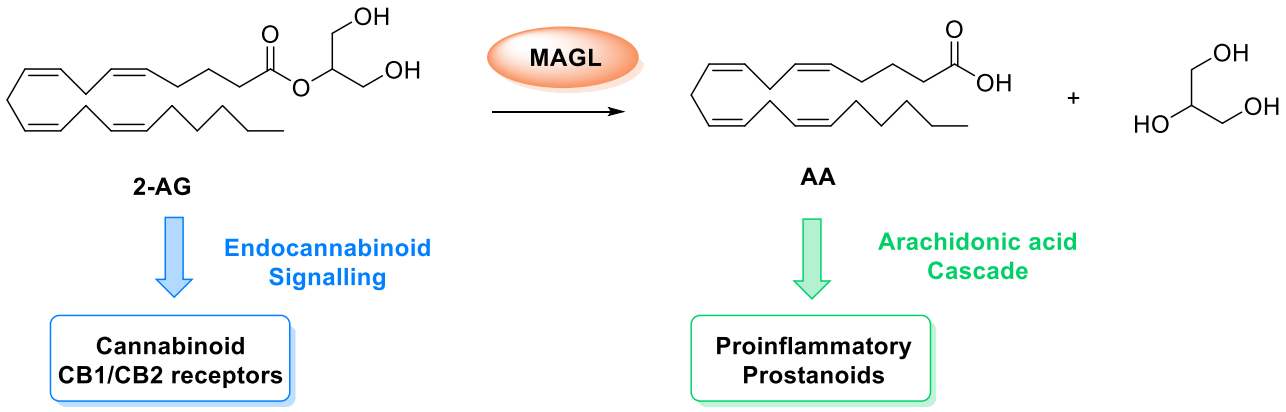


**Figure 24.** Metabolic and signalling pathways of 2-AG regulated by MAGL.

Both genetic and pharmacological blockade of MAGL has revealed a tissue distribution of this enzyme that is highly expressed in brain, liver, adipose tissue, intestine, lungs, and other tissues. In brain, MAGL is expressed in neurons, oligodendrocytes, astrocytes, and microglia [113]. Moreover, it is located in presynaptic regions at axon terminals in proximity of CB1.

This membrane-associated enzyme was purified from rat adipose tissue and cloned from mouse adipocytes by Karlsson in 1997 [114], while the first X-ray crystal structures of MAGL were independently reported by Lambert et al. and by Bertrand and co-workers in 2009 [115, 116]. Its binding site comprises three functional channels: the acyl chain binding pocket (ABP), the alcohol-binding channel, and the glycerol exit channel, which are able to accommodate the acyl chain, the glycerol moiety, and the leaving group, respectively [115]. At the end of ABP, which appears as a large hydrophobic tunnel, is the catalytic triad Ser122-Asp239-His269 of MAGL. Especially, the triad activates Ser122, which triggers a nucleophilic attack to the carbonyl of 2-AG, forming a complex with it. A cavity of the enzyme named 'oxyanion hole' stabilizes the developing negative charge on the carbonyl in the transition state in route to the tetrahedral intermediate. Finally, regeneration of the enzyme occurs due to the hydrolysis of the enzyme-substrate complex mediated by His269, thus expelling AA and glycerol. Interestingly, three cysteines (Cys201, Cys208, Cys242), located near the catalytic triad, were hypothesized to have a role in the stabilization of MAGL's active conformation and additionally interact with some substrates. [116, 117]. These cysteines were once considered as key amino acid residues for the development of selective MAGL inhibitors over other serine hydrolases [118].

Due to its role as 2-AG metabolizing enzyme, MAGL plays a critical role in the regulation of eCBs and eicosanoid signalling pathways [119, 120] and its inhibition induces a reduction of AA, the well-known precursor of proinflammatory prostanoids, and an increase of 2-AG, thus resulting in the decrease of neuroinflammation.

Although the translational potential of MAGL needs to be firmly clarified, some research results strongly support the role of MAGL in ameliorating NDs, either by direct or indirect mechanisms. In this regard, it has been demonstrated that MAGL knock-out mice showed improved synaptic plasticity and memory, suggesting that the absence of MAGL activity could positively affect higher brain functions inhibition [121].

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Moreover, several studies described that systemic administration of MAGL inhibitory compounds had a beneficial impact on memory loss in a mouse model of AD. MAGL inhibition also prevented neuroinflammation, neurodegeneration, maintained hippocampal integrity, and improved long-term synaptic plasticity, spatial learning, and memory in AD animals [122]. While inhibition of 2-AG metabolism mitigates Aβ neuropathology, it is still not clear whether inhibition of MAGL alleviates tauopathies.

Regarding PD, both pharmacological and genetic inactivation of MAGL improved the phenotype in the methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)/probenecid mouse model of PD. In detail, administration of an irreversible MAGL inhibitor prevented the MPTP-induced dopaminergic neuronal loss in the substantia nigra and attenuated dopaminergic neuronal terminal loss [123]. To support the potential of MAGL for controlling PD alterations, an independent study described that MAGL inhibitor KML29 (see below) also attenuated the MPTP/probenecid-induced striatal dopamine loss [124].

In light of these findings, MAGL can be considered a promising therapeutic target to drive the aberrant and networked pathways of NDs.

*5.1. MAGL inhibitors*

The determination of the crystal structure of MAGL has provided a comprehensive characterization of the active site and the catalytic mechanism of the enzyme and its interaction with the substrate, paving the way to the discovery of MAGL inhibitors. In the last decades, extensive efforts by both academic research groups and pharmaceutical companies were addressed for the development of MAGL inhibitors. Based on their mechanism of action, they can be classified into two main groups: i) irreversible inhibitors, that comprise cysteine -reactive agents and serine -reactive agents such as maleimides, disulfides, carbamates, ureas and arylthioamides; ii) reversible inhibitors. Among them, the most reported MAGL inhibitors are irreversible ones, as extensively reviewed elsewhere [125].

Herein, a short summary of representative MAGL inhibitors is given by focusing on the recently emerged network between MAGL activity and neurodegenerative-related pathways, which may be exploited in the future for the design of DMLs.

*5.1.1 Covalent inhibitors*

Among cysteine-reactive agents, maleimides, comprising a mercapto-specific function, represent the starting point for MAGL inhibitor development. Maleimide derivatives were shown to covalently and irreversibly target the sulfhydryl group of cysteine residues through a Michael addition, yielding a S-alkylated MAGL adduct. Saario *et al*. [126] reported a study on *N*-arachidonoylmaleimide (NAM, Figure 25) as a very potent inhibitor ($IC_{50}$ = 0.14 μM) and in a following paper Lambert [127] described the maleimide biphenyl derivative **40** (Figure 25), which showed moderate inhibitory activity and selectivity over FAAH ($IC_{50}$ (MAGL) = 0.79 μM vs $IC_{50}$ (FAAH) = 16.6 μM). Notably, the major drawback of the maleimide groups are their probable off-target effects due to the reaction with other cysteine-containing proteins, hampering their use for further functional studies or development as drug candidates. Then, the same research group also reported a study on disulfiram (**41**, Figure 25), a disulfide drug approved for alcoholism treatment, that also proved to inhibit human MAGL at submicromolar concentrations. ($IC_{50}$ = 0.36 μM) [128]. SAR studies performed on **41** indicated that *N*-substituted derivatives improved the activity and selectivity for human MAGL [129]. These derivatives were hypothesized to covalently interact with MAGL cysteine residues Cys208 and Cys242.

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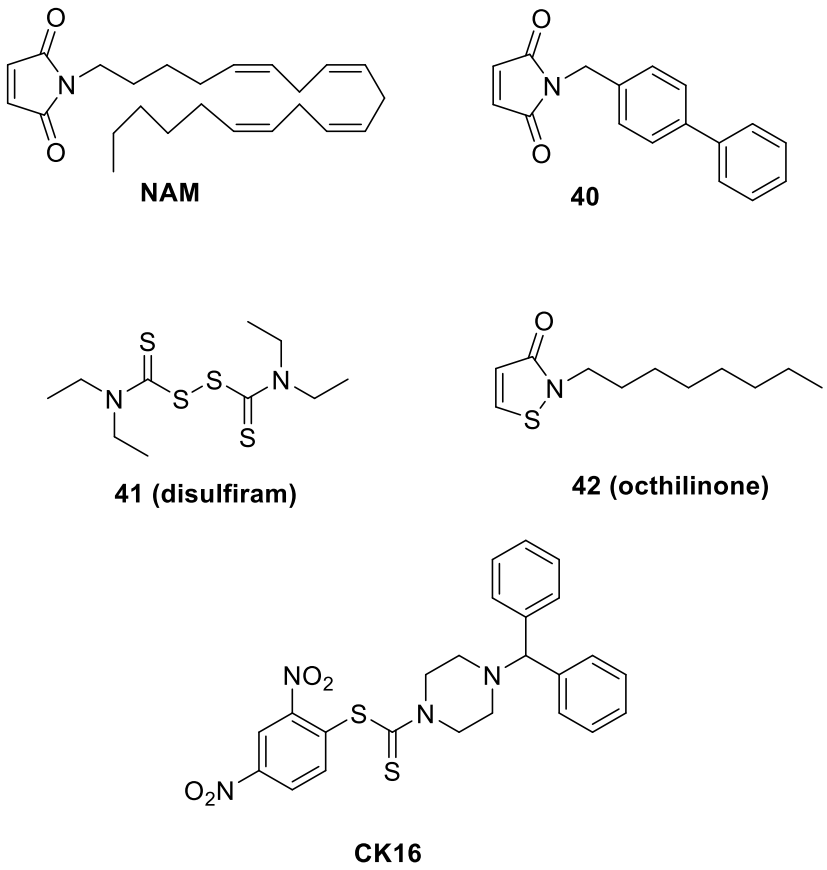


**Figure 25.** Representative cysteine-targeting MAGL inhibitors.

Isothiazoline derivatives are considered an interesting scaffold for the design of novel MAGL inhibitors acting on Cys residues. In this context, King and co-workers investigated a series of sulfhydryl-specific molecules and described octhilinone (**42**, Figure 25) to block MAGL activity ($IC_{50}$ = 88 nM) through a partially reversible mechanism, as determined by dilution assay studies [130]. Moreover, a series of arylthioamides was reported by Popaert *et al*. showing moderate inhibitory activity and selectivity over FAAH. Among these, CK16 ($IC_{50}$ = 0.35 µM, Fig. 25) proved to be the most potent and selective inhibitor (more than 2800-fold over FAAH) [131]. This compound, characterized as an irreversible inhibitor, probably interacts with Cys208 and Cys242.

Carbamates can be considered as the most studied class of MAGL inhibitors, *pseudo*-irreversibly interacting with the catalytic residue Ser122, followed by the release of a leaving group and the formation of a stable adduct between the enzyme and the inhibitor. URB602 (Figure 26) was the first generation of MAGL O-aryl carbamate developed by Hohmann and co-workers. It showed a weak inhibitory profile against MAGL ($IC_{50}$ = 28 µM) [132] and cross-reactivity with FAAH, making URB602 unsuitable for additional MAGL functional studies. Moreover, dialysis experiments suggested that URB602 is not a full irreversible agent, but partially reversibly binds to MAGL. To optimize carbamates as selective scaffolds for MAGL inactivation over FAAH, Cravatt and co-workers identified the first selective and *in vivo* active MAGL inhibitor JZL184 (Figure 26, $IC_{50}$ (MAGL) = 6 nM *vs* $IC_{50}$ (FAAH) = 4 µM) [133], devoid of off-target effects towards CBRs and other serine hydrolase enzymes. Discovery of JZL184 was regarded as one of the most important breakthroughs for MAGL inhibitors development, and it accelerated the understanding of MAGL's physiological roles.

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Afterwards, Chen et al. showed that JZL184 significantly reduced neuropathology and improved synaptic and cognitive functions in 5XFAD APP transgenic mice, strongly supressing the production and accumulation of Aβ [134]. A recent study also showed that JZL184 significantly reduced proinflammatory cytokines, phosphorylated GSK3β and tau, cleaved caspase-3, and phosphorylated NF-kB in P301S/PS19 mice, tau mouse model of AD. Importantly, tau transgenic mice treated with JZL184 displayed improvements in spatial learning and memory retention [135].

Other compounds belonging to the carbamate class are KML29 [136], JW651, MJN110, which could be promising agents for NDs (Figure 26). ABX-1431 (Figure 26), a first-in-class irreversible inhibitor of MAGL developed by Grice and co-workers, able to suppress pain behaviour in rat formalin pain model, is now entering clinical phase II studies for neurological disorders and other diseases [137]. Recently, McAllister *et al*. at Pfizer Worldwide Research and Development, reported PF-06795071 (Figure 26), a potent and selective covalent MAGL inhibitor, designed aiming at identifying an optimized leaving group capable of improving some important features, mainly potency, selectivity and solubility, with respect to the hexafluoroisopropanol leaving group, a recurrent motif of covalent MAGL inhibitors [138]. Indeed, this group, also present in ABX-1431, conferred a satisfactory potency and selectivity profile, but showed high lipophilicity that impacted on solubility and brain penetration. They found that the trifluoromethyl glycol leaving group was able to impart good potency and selectivity towards different serine hydrolases, improve solubility, and increase CNS exposure. PF-06795071 showed an appropriate brain penetration, a reduction in brain inflammation, and a sustained 2-AG elevation *in vivo*, together with a good safety profile.

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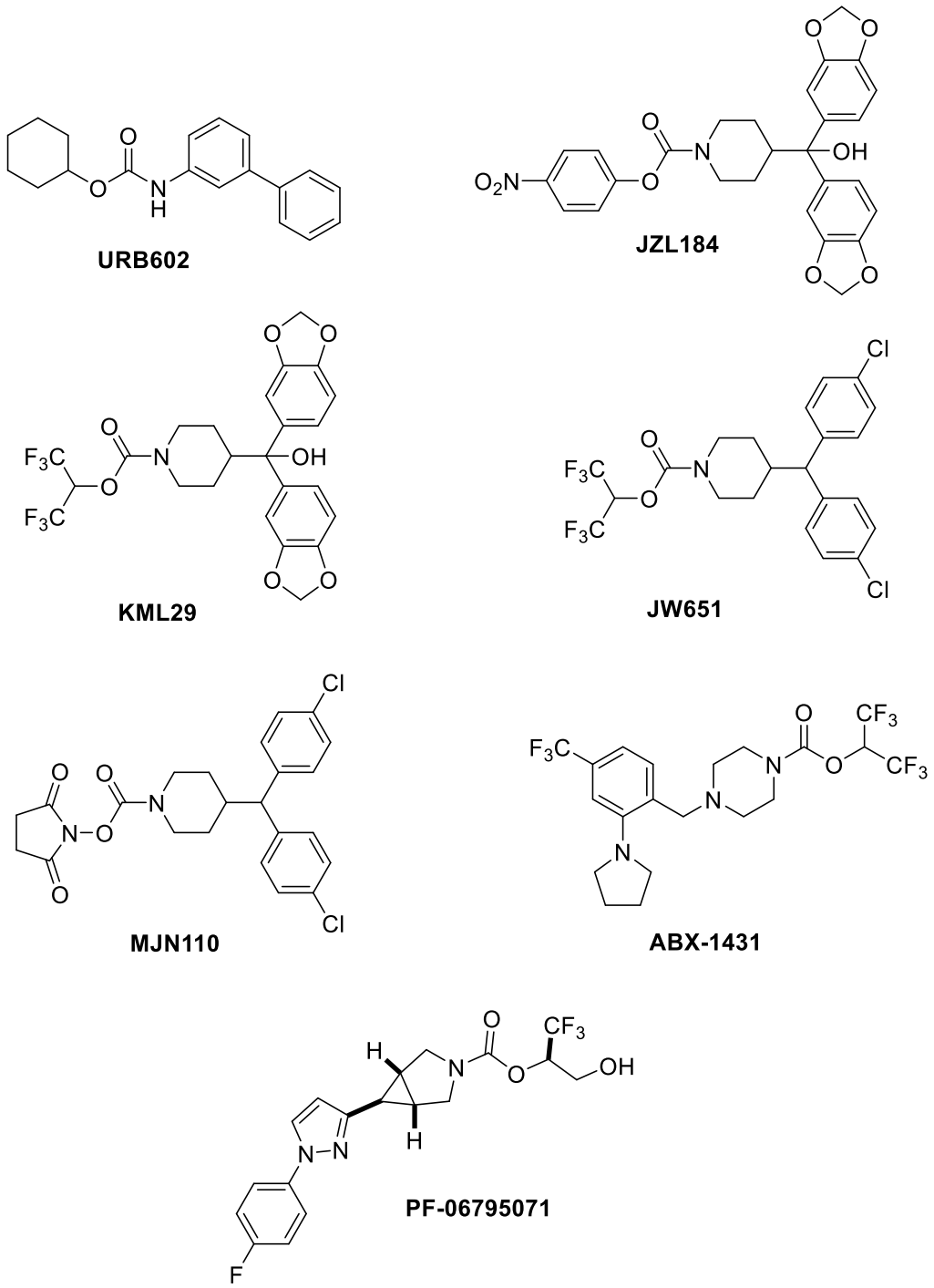


**Figure 26.** Representative carbamate MAGL inhibitors.

*5.1.2. Non-covalent inhibitors*

The permanent inactivation of MAGL caused by covalent inhibitors can induce functional ECS antagonism, leading to pharmacological tolerance and receptor desensitization, progressively impairing the inhibitor benefits and leading to undesired effects [120]. This drawback has greatly limited the development of MAGL inhibitors, unveiling the urgent need for the design of potent and selective reversible inhibitors. In

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recent years, several research groups have explored this opportunity [139, 140], mainly related to cancer therapy, since MAGL also plays a significant role in malignancies [125]. Regarding NDs, the group of Maria Lopez-Rodriguez, starting from the previously reported unselective MAGL/FAAH inhibitors **43** and **44** (Figure 27) [141], performed a structural exploration study aiming at the identification of potent, selective, and reversible MAGL inhibitors [142]. Compound **45** (Figure 27) emerged as the most interesting, showing *in vivo* activity and the ability to ameliorate the progression of MS in a mouse model, without inducing unwanted side effects mediated by CB1 activation.

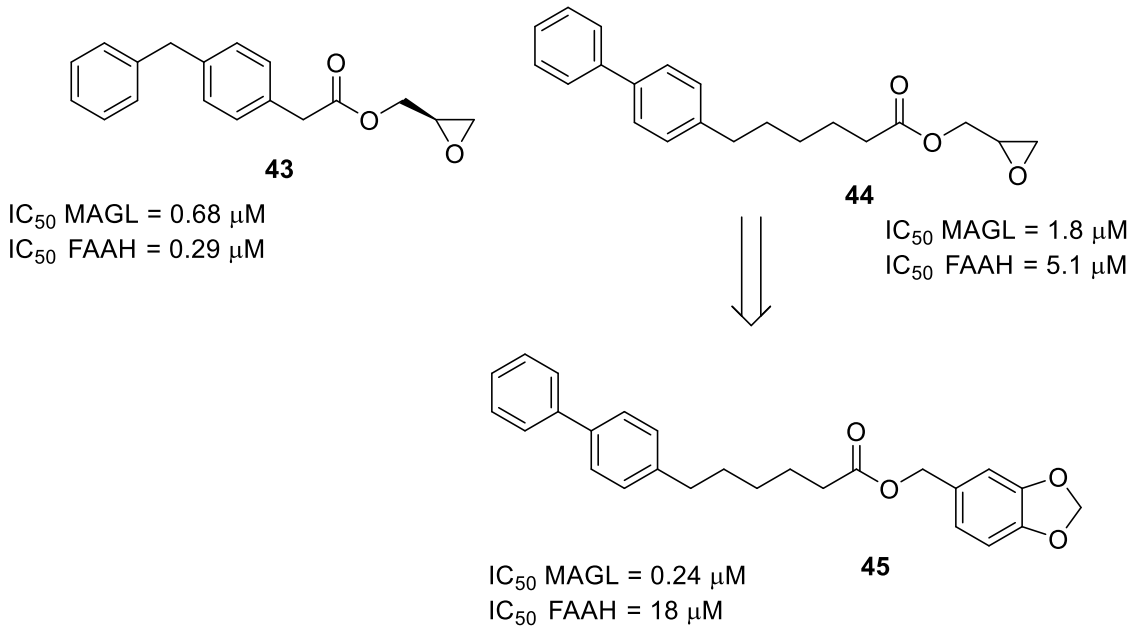


**Figure 27.** Reversible selective MAGL inhibitors for MS

More recently, Aida *et al*. from Takeda Pharmaceuticals, applying a high-throughput screening campaign, identified the pyrrolidinone derivatives **46** and **47** (Figure 28) [143] as hit compounds. Taking advantage of the previously reported co-crystal structure of the amide derivative **48** with MAGL enzyme [144], they adopted a structure-based drug design approach to identify novel piperazinyl pyrrolidin-2-one derivatives. A further optimization study led to the reversible MAGL inhibitor **(R)-49** (Figure 28) endowed with low nanomolar potency. This compound proved to decrease AA levels and to increase 2-AG concentration in the brain following oral administration in mice, proving to be a promising candidate for NDs treatment.

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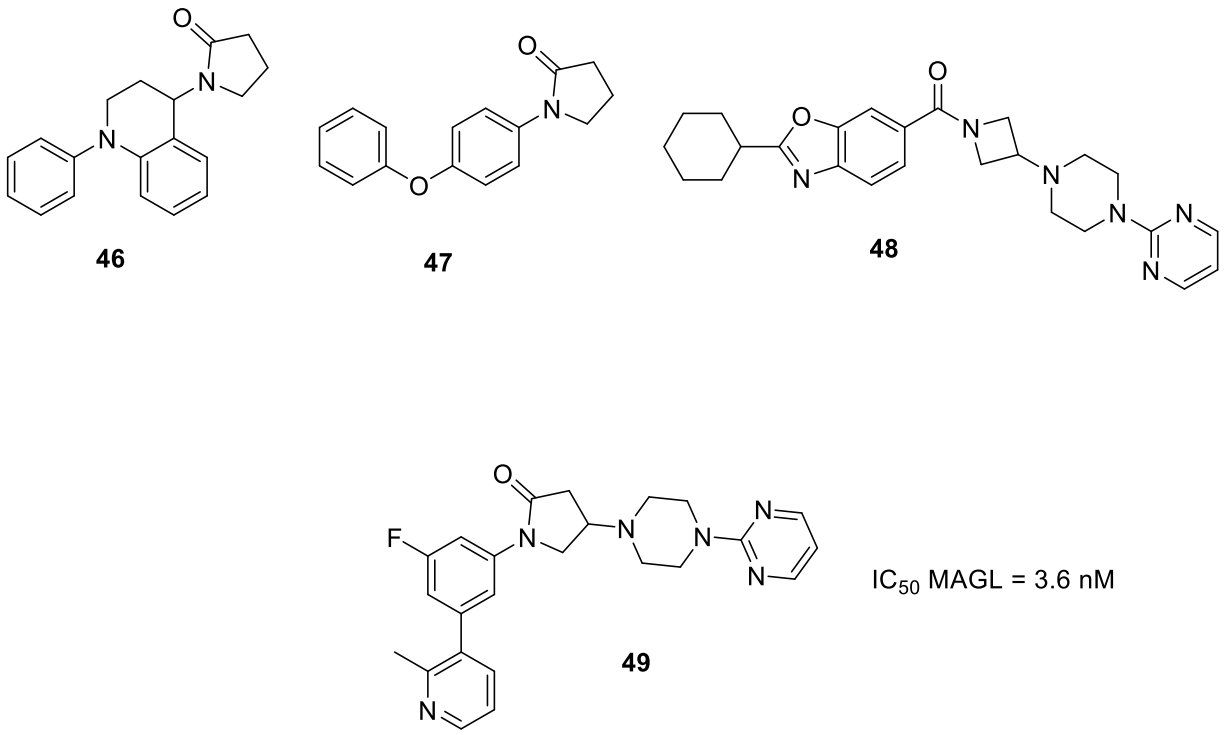


**Figure 28.** Takeda pyrrolidinone derivatives as MAGL potent selective inhibitors.

Zhu and co-workers from Janssen Research & Development, aimed at improving the metabolic stability of their hit compound **48**, reported in 2020 a structural optimization study, which led to the discovery of a novel series of diazetidinyl diamides as potent reversible MAGL inhibitors [145]. Among their series, compounds **50** and **51** (Figure 29) showed promising activity and selectivity and were also endowed with good brain penetration after oral administration in vivo. These compounds can be regarded as tools to validate MAGL inhibition as a therapeutic strategy to treat NDs.

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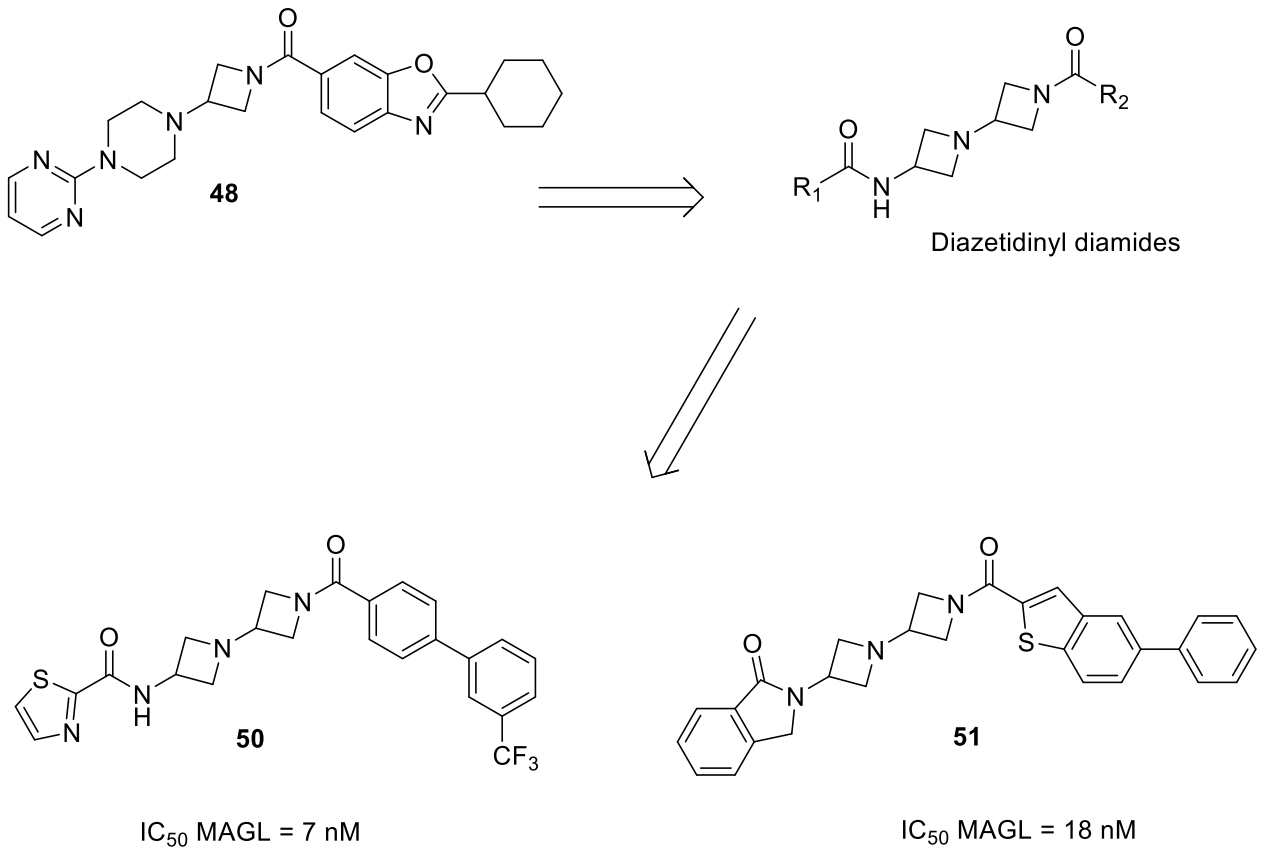


**Figure 29.** MAGL inhibitors developed at Janssen.

## 6. CONCLUSIONS AND FUTURE PERSPECTIVES

Over the last years, the increasing significance of ECS role in NDs has been confirmed by the relevant number of papers and reviews dealing with this topic, which however, also highlight the complex regulation mechanism of this system. Indeed, despite the multiple interaction modes and the number of molecules acting on ECS recently designed and synthesized, no compound has currently reached the market for NDs treatment. In particular, the development of clinically useful CB2R agonists, still considered one of the most attractive approaches to face NDs, appears very challenging, mainly due to the predominance of CB2R in immune cells and the derived immunosuppression which may occur following chronic administration. Therefore, the design of clinically useful drugs should explore novel therapeutic strategies for modulating CB2R through new mechanisms of action.

This is particularly true for the MTDL approach, in which the designed molecule is assumed to act on different targets, often belonging to different protein families [146], characterized by conflicting structural requirements. In this respect, the search for diverse receptor modulating modes may represent a winning opportunity. Allosterism and biased signalling mechanisms at CB2R may offer new promises for the improving of CB2R-targeted therapies [38, 52, 147]. Moreover, as abovementioned, bivalency may also represent a useful tool to investigate CBRs functions and their crosstalk with other physiological systems.

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Further exploiting polypharmacology and aiming at overcoming current treatment limitations, a recent paper proposed new emerging targets to be modulated together with CB2R, in particular histone deacetylases and σ receptors [52]. Modern medicinal chemistry strategies, mainly computational polypharmacology and molecular hybridization, were suggested to reach this goal, thanks to the availability of 3D structures of the selected targets and the possibility to identify common pharmacophoric fragments.

Interestingly, CB1 and CB2 are not the only receptors involved in modulating ECS, and the potential of GPR55 [148] and GPR18 [10] has recently been explored, setting the stage for further avenues to go in finding effective drugs.

An alternative strategy is the modulation of ECS through inhibition of the foremost metabolizing enzymes FAAH and MAGL. This approach could be better suited for the treatment of NDs, allowing prolonged CBRs stimulation while avoiding the adverse side effects associated with direct CB1R stimulation by exogenous cannabinoids. To date, only one MAGL inhibitor has been tested in clinical trials; while inhibitors of FAAH have advanced to phase II clinical trials for diverse indications, showing promising low toxicity and a distinctive ability to regulate emotional disorders [124]. By contrast, conflicting results were reported in PD, where MAGL inhibition seems to be superior. While it is too early to assume whether FAAH and MAGL inhibitors will emerge as effective treatments, these reports allow to consider these enzymes as privileged targets in the development of MTDLs for AD, also considering that they belong to the serine hydrolase family as ChEs, which may facilitate MTDL design, compared to the identification of a compound acting on both a GPCR and an enzyme.

Finally, numerous preclinical evidences have suggested a major role for eCBs in regulating adult neurogenesis [149]. In particular, an improved CBRs activity, obtained by increasing eCBs load or by inhibiting their degradation, was seen to induce neurogenic effects and the rescue of phenotypes in animal models of different neurological disorders, paving the way for new unexpected future perspectives.

## 7. LIST OF ABBREVIATIONS

ECS = Endocannabinoid System

CNS = Central Nervous System

eCBs = Endocannabinoids

AEA = Anandamide

2-AG = 2-ArachidonoylGlicerol

GPCR = G-Protein Coupled Receptor

CB1R = CB1 Receptor

CB2R = CB2 Receptor

PPAR = Peroxisome Proliferator-Activated Receptor

TRPV1 = Transient Receptor Potential cation channel subfamily V member 1

NAPE-PLD = N-Acyl-PhosphatidylEthanolamine-PhosphoLipase D

DAGL = DiAcylGlycerol Lipase

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FAAH = Fatty Acid Amide Hydrolase

MAGL= MonoAcylGlycerol Lipase

NMDAR = N-Methyl-D-Aspartate Receptor

CBRs = Cannabinoid Receptors

THC = trans-Δ9-TetraHydroCannabinol

CBD = CannaBiDiol

CBC = CannaBiChromene

CBG = CannaBiGerol

AD = Alzheimer's Disease

ND = Neurodegenerative Disease

PD =Parkinson's Disease

HD = Huntington's Disease

MS = Multiple Sclerosis

ALS = Amyotrophic Lateral Sclerosis

Aβ = Amyloid-beta

SAR = Structure-Activity Relationship

SAfiR = Structure-Affinity Relationship

MTDL = MultiTarget-Directed Ligand

ChE = Cholinesterase

AChE = AcetylCholinesterase

BChE = ButyrylCholinesterase

DML = Designed Multiple Ligand

BACE-1 = Beta-site Amyloid precursor protein Cleaving Enzyme 1

CAS = Central Anionic Site

PAS = Peripheral Anionic Site

MAO-B = MonoAmino Oxidase B

ABHD1 = α,β-Hydrolase Domain

AA = Arachidonic Acid

ABP = Acyl-chain Binding Pocket

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MPTP = Methyl-4-Phenyl-1,2,3,6-TetrahydroPyridine

NAM = N-ArachidonoylMaleimide

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## 9. CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise

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